UNIVERSITY OF EDINBURGH

The physiological role of the endothelin system in the maintenance of vascular tone in man

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DECLARATION

This thesis represents research undertaken in the University of Edinburgh's Clinical Pharmacology Unit and Research Centre at the Western General Hospital, Edinburgh. The substantial part of the work described has been my own and carried out during the period between 1995 and 2000 whilst I was a Senior Research Nurse. I have been fortunate in gaining the advice and assistance of many colleagues, and they have been formally acknowledged. The majority of the work has been published in peer reviewed journals: see Bibliography. The thesis has not been accepted in any previous applications for a degree and all sources of information have been acknowledged.

Fiona Elizabeth Strachan

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ABSTRACT

Since its discovery in 1988, endothelin-1 (ET-1) has emerged as a potential therapeutic target in the treatment of cardiovascular disease. The powerful vasoconstrictor effects of ET-1 are primarily mediated via the vascular smooth muscle cell, ET-1 selective ETA receptor, while the non-isopeptide selective ETB receptor, situated on endothelial cells, mediates vasodilatation through generation of nitric oxide and prostacyclin. ETB receptors have also been described on vascular smooth muscle cells, although their contribution to ET-1 mediated vasoconstriction remains to be confirmed.

The aims of this thesis were to assess the relative contribution of each receptor subtype to ET-1 mediated vasoconstriction and, in particular, investigate the endogenous effects of the ETB receptor on vascular tone. In a series of clinical studies in healthy men, the effects of endothelin receptor agonists and antagonists on local and systemic measures of vascular tone were assessed using standard techniques. All studies were carried out with the approval of the local Research Ethics Committee and with the written informed consent of each subject. Locally active doses of ET receptor agonists were used in combination with locally and systemically active doses of ET receptor antagonists. Responses in capacitance vessels were assessed by measurement of changes in dorsal hand vein size by a standard displacement technique. Responses in resistance vessels were assessed by measurement of changes in forearm blood flow by venous occlusion plethysmography. Changes in cardiac function were measured non-invasively by a standard bioimpedance method.

Constriction in capacitance and resistance vessels was demonstrated in response to locally active infusion of the non-selective ET receptor agonist ET-1 and the ETB receptor selective agonists sarafotoxin S6c (SFTX6c) and BQ-3020, supporting a potential role for the ETB receptor in ET-1 mediated vasoconstriction. In capacitance vessels, constriction to ET-1 but not SFTX6c was attenuated during co-infusion of the ETA receptor selective antagonist BQ-123, in contrast constriction to SFTX6c but not ET-1 was attenuated during co-infusion of the ETB receptor selective antagonist BQ-788. In addition to the vasoconstriction described with low dose infusion of ETB receptor selective agonists, vasodilatation, which appeared to be mediated largely by nitric oxide through ETB receptor stimulation, was demonstrated with high dose intra-arterial infusion of the ETB agonist SFTX6c. The repeatability of the forearm blood flow response to intra-arterial infusion of ET-1 was assessed and, using this response as a model, a pharmacologically active dose range of the ETA receptor selective antagonist BMS-193884 and the non-selective ETA/ETB receptor antagonist MSD L-753,037 was identified.

Although ETB receptor mediated pharmacological effects can be demonstrated by administration of ETB receptor agonists, the endogenous physiological effects of the ETB receptor are perhaps better demonstrated by ETB receptor selective antagonists. Local and systemic vasoconstriction were demonstrated in response to locally and systemically active infusion of the ETB receptor selective antagonist BQ-788. Both ETA receptor selective and combined ETA/ETB receptor antagonism resulted in significant forearm vasodilatation. However, the local vasodilator effects of the ETA receptor selective antagonist BQ-123 were attenuated in the presence of the selective ETB receptor antagonist BQ-788. These results confirm the importance of ET-1 in the maintenance of vascular tone and indicate that, in healthy blood vessels at least, the vasodilator effects of non-selective ETA/ETB receptor antagonism are less than those of ETA receptor selective antagonism.

The results of these investigations have provided valuable information on the relative contribution of the ETA and ETB receptors to the physiological effects of ET-1 in the vasculature. In addition they have developed and characterised a series of tools for future clinical studies. Further investigation of the integrated cardiovascular effects of selective and non-selective ET receptor antagonists is required to confirm which approach will be the more effective in the clinical setting.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

FIGURES

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ABBREVIATIONS

ACE	Angiotensin Converting Enzyme
ANOVA	Analysis of variance
AUC	Area under the curve
cGMP	Cyclic guanylate monophosphate
CO	Cardiac output
CI	Cardiac index
ECE	Endothelin converting enzyme
EDCF	Endothelium-derived constricting factor
EDHF	Endothelium-derived hyperpolarising factor
EDTA	Ethylene diamine tetraacetic acid
ET	Endothelin
ET_A	Endothelin type A receptor
ET_B	Endothelin type B receptor
FBF	Forearm blood flow
IC_{50}	Inhibitory concentration to reduce response by 50%
K_i	Inhibition constant
K_D	Dissociation constant
L-NMMA	L-N^G-monomethyl arginine
MAP	Mean arterial pressure
NO	Nitric oxide
NOS	Nitric oxide synthase
PGI_2	Prostaglandin
SEM	Standard error of the mean
SFTX6c	Sarafotoxin S6c
TPVR	Total peripheral vascular resistance
TPVRI	Total peripheral vascular resistance index

Chapter 1

Introduction

1.1 The maintenance of vascular tone

Vascular tone is an important determinant of peripheral vascular resistance and, in turn, blood pressure. Blood pressure is controlled through the interaction of a number of physiological mechanisms and is influenced primarily by changes in cardiac output and peripheral vascular resistance. The regulation of peripheral vascular resistance is important in maintaining blood pressure in situations where there are changes in cardiac output, for example during exercise.

The radius of a vessel is an important determinant of the resistance within that vessel, with vasodilatation acting to reduce the resistance and vasoconstriction acting to increase resistance. Vascular resistance is abnormally increased in arterial hypertension and in chronic heart failure. A number of therapeutic approaches in cardiovascular disease, including ACE inhibition, act to reduce vascular resistance through vasodilatation.

1.1.1 Circulating mediators of vascular tone

Short term regulation of vascular tone is achieved by neuronal regulation through baroreflexes and autonomic reflexes via the sympathetic neurotransmitters, adrenaline and noradrenaline (catecholamines) and the parasympathetic neurotransmitter, acetylcholine. In addition, neurohumoral factors such as the renin-angiotensin system, ADH or arginine vasopressin and ANP play a role in the longer term control of vascular tone through regulation of blood volume and direct effects on blood vessels.

1.1.2 Local mediators of vascular tone

In addition to the central mediators described above, a number of local mediators are involved in the regulation of vascular tone. A number of these mediators are released by the endothelium, which was once thought to represent merely an inert lining to

blood vessels. The importance of the endothelium in the regulation of vascular tone is now widely recognised (Vane, et al., 1990).

Endothelial stimulation results in the immediate release of a number of vasodilator mediators including prostacyclin (Moncada, et al., 1976); nitric oxide (Ignarro, et al., 1987; Palmer, et al., 1987) and endothelium-derived hyperpolarising factor (EDHF). The recognition of an endothelium-derived constrictor factor (EDCF) (Hickey, et al., 1985), which has subsequently been identified as endothelin-1 (ET-1) (Yanagisawa, et al., 1988), demonstrates the ability of the endothelium to provide a counter-regulatory tonic action on the adjacent vascular smooth muscle.

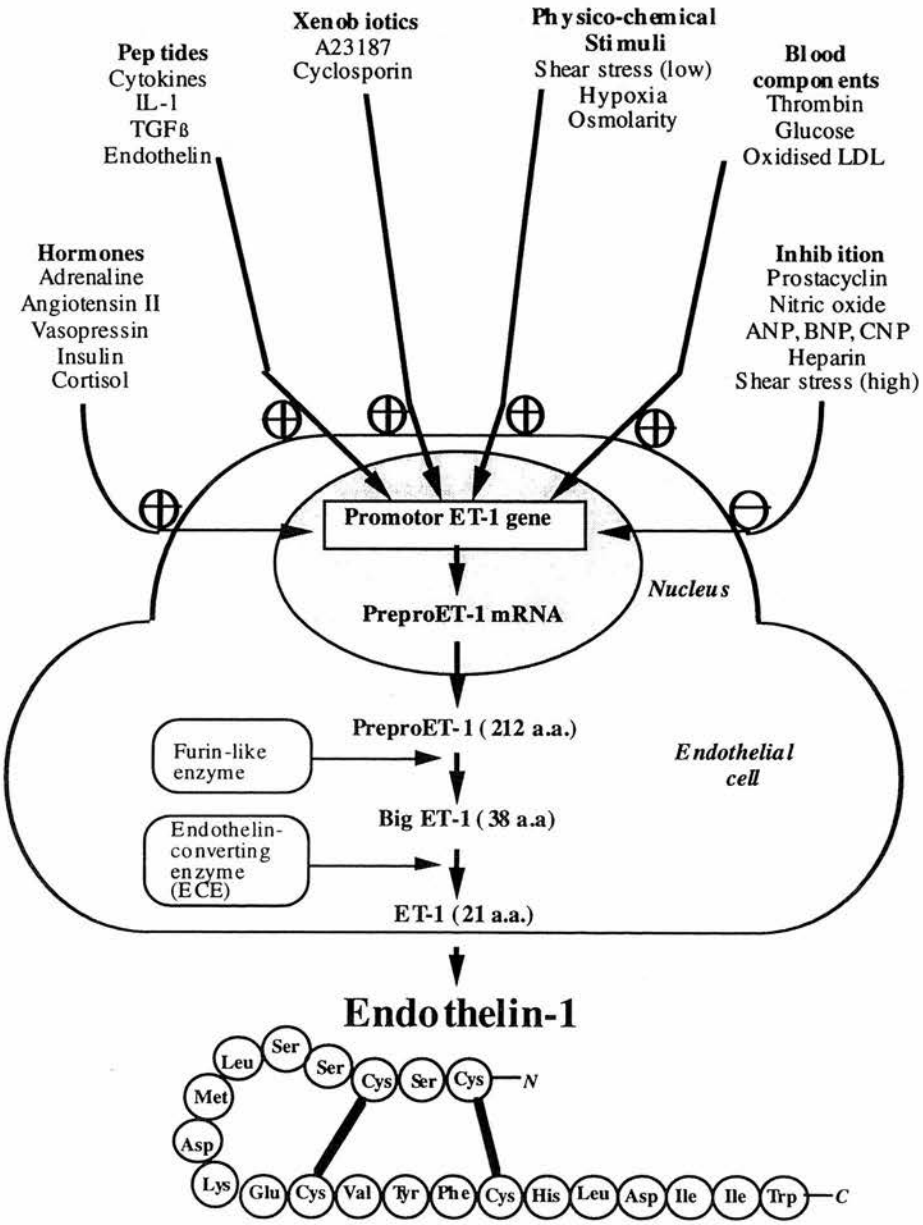
Both nitric oxide and ET-1 are released continuously by the endothelium to regulate basal vascular tone (Haynes and Webb, 1994; Vallance, et al., 1989). Thus, the endothelium plays a crucial role in the local paracrine regulation of vascular tone, blood flow and blood pressure. Disruption of normal endothelial function can, therefore, have a marked influence on the local and systemic haemodynamic balance, and ultimately tissue perfusion. Indeed, endothelial dysfunction is now recognised as an important factor in the pathophysiology of cardiovascular disease (Casino, et al., 1995; Luscher, 1992; Panza, et al., 1990) and the endothelium has been the target of recent treatments in cardiovascular disease including aspirin, nitrates and angiotensin-converting enzyme (ACE) inhibitors.

1.2 Endothelin

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide generated by the endothelium (Yanagisawa, et al., 1988). It is one of a family of three related peptides; ET-1, endothelin-2 (ET-2) and endothelin-3 (ET-3), encoded in three distinct genes (Inoue, et al., 1989). As ET-1 is the predominant isoform produced by the endothelium, it is likely to be the most important in the regulation of vascular tone. Endothelin-1 is generated from its precursor, big endothelin-1, through the actions of an endothelin converting enzyme (ECE). Generation of ET-1 is regulated at a transcriptional level and is influenced by a number of factors (Figure 1.1).

The discovery of ET-1, originally described as EDCF (Hickey, et al., 1985), followed closely on the identification of the endothelium-derived relaxing factor (EDRF) as nitric oxide (Palmer, et al., 1987) and these two mediators appear to act in opposition to regulate vascular tone and blood pressure. Even in their original Nature paper, Yanagisawa and colleagues raised the possibility that ET-1 might be involved in the maintenance of blood pressure and the genesis of hypertension (Yanagisawa, et al., 1988). There are now considerable preclinical and clinical data to support this view.

Figure 1.1 Factors that alter endothelin-1 (ET-1) synthesis and the pathway for endothelin-1 generation: see text for details (Section 1.2.1). IL-1=interleukin-1; TGFß=transforming growth factor ß; LDL=low-density lipoprotein; ANP, BNP, CNP=atrial, brain, and c-type natriuretic peptides.

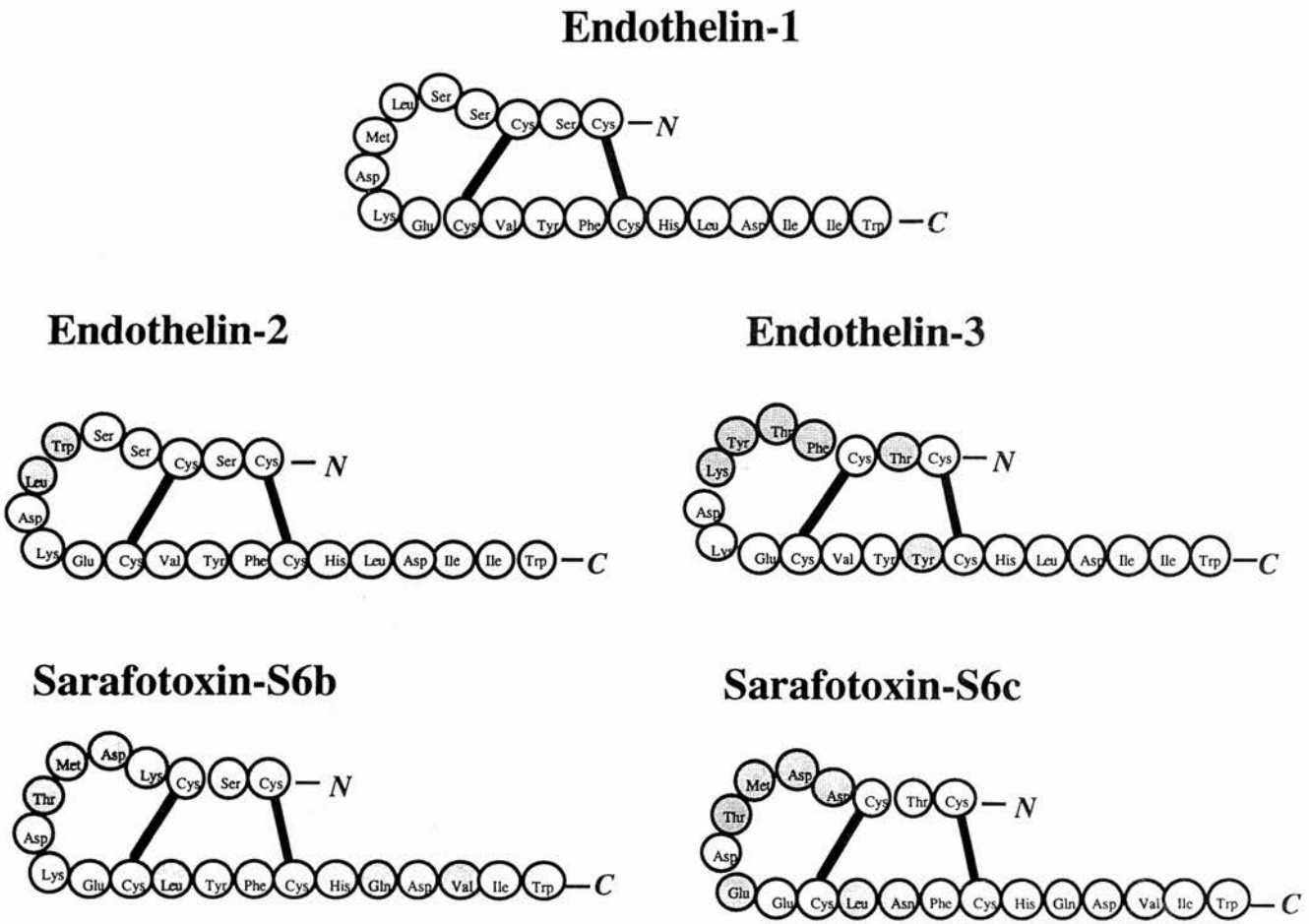


1.2.1 Endothelin genes and regulation

Endothelin-1 was originally identified in the culture medium of porcine aortic endothelial cells (Yanagisawa, et al., 1988). It is now recognised to be a member of a family comprising three isoforms (Inoue, et al., 1989): ET-1, ET-2 and ET-3 [Figure 1.2]. Each isoform contains 21 amino-acids, two intra-chain disulphide bonds linking paired cysteine residues, and a conserved C-terminal sequence necessary for biological activity (Inoue, et al., 1989). This structure is unique among the mammalian peptides but is shared by the sarafotoxins (Figure 1.2), snake venom peptides from the Israeli burrowing asp, *Actractaspis engaddensis*. One of the sarafotoxins, sarafotoxin S6c (Williams, et al., 1991), has proven particularly valuable as a pharmacological probe with a high selectivity for the ET_B receptor.

Within the human genome, the endothelins are each represented by a separate gene encoding a specific precursor for the mature isoform (Inoue, et al., 1989). Currently, regulation of endothelin synthesis is thought to be primarily at the level of gene transcription, with *de novo* production and release occurring in response to endothelial cell stimulation by a number of extracellular factors. Factors acting at this level to stimulate ET-1 synthesis (Figure 1.1) include insulin, thrombin, low density lipoprotein, angiotensin II, vasopressin and ET-1 (Benatti, et al., 1994; Boulanger, et al., 1992; Emori, et al., 1992; Emori, et al., 1991). These factors are thought to act via protein kinase C (PKC). In contrast, other factors, including nitric oxide (Boulanger and Luscher, 1990), natriuretic peptides (Kohno, et al., 1992), dilator prostanoids (Prins, et al., 1994) and thrombin (Boulanger and Luscher, 1990) inhibit ET-1 generation by promoting production of cyclic GMP (cGMP). Low levels of shear stress (1.8 dyne/cm^2) are thought to enhance endothelial ET-1 release by PKC activation (Wang, et al., 1993) wherease high levels of shear stress (>6 dyne/cm^2) inhibit ET-1 mRNA transcription (Malek, et al., 1993) possibly through nitric oxide.

Figure 1.2 Diagramatic representation of endothelin and sarafotoxin isoforms. Shaded circles represent amino acids that differ from ET-1.



1.2.2 Endothelin precursors

The product of human ET-1 gene transcription is preproendothelin-1, a peptide of 212 amino acid residues. After removal of a short secretory sequence, preproendothelin-1 undergoes cleavage by a dibasic pair specific endoprotease to generate the 38 amino-acid peptide, 'big endothelin-1' (Yanagisawa, et al., 1988). Subsequent conversion to the mature, biologically active peptide, ET-1, occurs through the action of a specific endothelin converting enzyme (ECE). The gene encoding ET-1 can be detected in a wide variety of tissues, including the endothelial cells, heart, lung, brain, kidney, pancreas and spleen. Big ET-1, ET-1 and ET-3 are normally present in plasma at picomolar concentrations that are probably insufficient to exert a direct influence on vascular tone. Indeed, ET-1 is generally thought to be a paracrine and autocrine mediator rather than an endocrine hormone and its secretion by endothelial cells is largely abluminal, towards the adjacent vascular smooth muscle (Wagner, et al., 1992). As a result, plasma concentrations of ET-1 are not always an accurate reflection of functional activity.

1.2.3 Endothelin converting enzymes

The physiologically relevant ECE is thought to be a membrane-bound, zinc containing metalloprotease that is inhibited by the neutral endopeptidase (NEP 24.11) inhibitor phosphoramidon (Ikegawa, et al., 1991) but not by selective NEP inhibitors such as thiorphan or ketalorphan (Mattera, et al., 1993).

At least two ECE isoforms have been identified ECE-1 (Xu, et al., 1994) and ECE-2 (Emoto and Yanagisawa, 1995). ECE-1, has a neutral pH optimum and is inhibited by phosphoramidon in micromolar concentrations. ECE-2, is also inhibited by phosphoramidon, but in nanomolar concentrations, and, in contrast to ECE-1, has an acidic pH optimum. The distribution of these enzymes also appears to be different. ECE-1 is widely distributed, with abundant expression in endothelial cells (Xu, et al.,

1994). ECE-2 is abundantly expressed in neural tissues, in contrast, ECE-1 is not found in neural tissues, suggesting that ECE-2 may be the major ECE in neurons, glia and selected neuroendocrine cells (Emoto and Yanagisawa, 1995). Both isoenzymes convert big ET-1 in preference to big ET-2 or big ET-3 (Xu, et al., 1994). Although further isoforms have subsequently been described (Turner, et al., 1998), it is not yet clear which isoform is most important in the pathophysiology of cardiovascular disease.

Although phosphoramidon effectively inhibits ET-1 formation (Emoto and Yanagisawa, 1995; Mattera, et al., 1993; Plumpton, et al., 1995; Xu, et al., 1994), its therapeutic potential is limited by its low potency and by its lack of selectivity for ECE. As yet there are no selective ECE inhibitors available for clinical use but there are currently several in preclinical development (Descombes, et al., 1995).

1.2.4 Endothelin clearance and degradation

Intravenously administered [^{125}I]ET-1 has a plasma half-life of less than 1 min in anaesthetised rats, most of the ET-1 being rapidly taken up by the lungs and kidneys (Anggard, et al., 1989; DeNucci, et al., 1988). In humans, ET-1 is rapidly cleared from the circulation by the renal, pulmonary and splanchnic circulation (Gasic, et al., 1992; Weitzberg, et al., 1991). Extraction of ET-1 follows binding to cell surface receptors, which are then internalised, allowing degradation to be carried out within the cell (Anggard, et al., 1989). Soluble proteases found in endothelial and vascular smooth muscle cells may be involved in intracellular ET-1 degradation (Jackman, et al., 1992; Jackman, et al., 1993). The endothelins can also be degraded by NEPs (NEP 24.11) on venous and arterial endothelial cell membranes (Llorens-Cortes, et al., 1992).

There is evidence to suggest that the ETB receptor is involved in clearance of ET-1 (Fukuroda, et al., 1994). Although low affinity ET_B binding sites that might serve this purpose have been found in arteries and veins (Gray, et al., 1994; Teerlink, et al., 1994) an exact site for this clearance receptor has not been confirmed.

1.3 Endothelin receptors

The endothelins act on two receptor subtypes in humans, ET_A (Arai, et al., 1990) and ET_B (Sakurai, et al., 1990) receptors, which have been characterised on the basis of their pharmacology (Table 1.1). The ET_A receptor is characterised by its high (subnanomolar) affinity for ET-1 and ET-2 and its 70 - 100 fold lower affinity for ET-3, while the ET_B receptor has a high and equal affinity for all 3 isopeptides.

1.3.1 Endothelin receptor subtypes

Shortly after its initial description (Yanagisawa, et al., 1988), specific binding sites for endothelin were identifed (Kloog, et al., 1989; Watanabe, et al., 1989) and the genes encoding the ET_A and ET_B receptor subtypes were cloned and characterised (Arai, et al., 1990; Sakurai, et al., 1990). Although there may be further functional hetrerogeneity (Bax and Saxena, 1994), analysis of human genomic DNA, with probes specific for the human ET_A and ETB receptors, reveals only two endothelin receptor genes (Sakamoto, et al., 1991). Therefore, any genes encoding other endothelin receptors within the human genome must have low sequence similarities to the known human endothelin receptor genes. There has been a recent tendency to sub-classify the ET_B receptor on the basis of responses to selective agonists and antagonists (Bax and Saxena, 1994) but this currently cannot be justified on a molecular basis.

Table 1.1 Pharmacological characterisation of endothelin receptors.

	ET_A	ET_B	
Agonist potency	ET-1>ET-2>>ET-3	ET-1=ET-2=ET-3	
Tissue	Vascular smooth muscle	Endothelium	Vascular smooth muscle
Action	Vasoconstriction	Vasodilatation	Vasoconstriction
Agonists	No selective agonists	ET-3 Sarafotoxin S6c BQ-3020	
Antagonists	BQ-123	BQ-788	

A putative ET_C receptor subtype, relatively selective for ET-3, has been cloned from *Xenopus laevis* dermal melanophores (Karne, et al., 1993). A novel ET-1 selective receptor has also been described on follicular membranes of *Xenopus laevis* oocytes (Kumar, et al., 1993) and from *Xenopus laevis* heart (Kumar, et al., 1994). This ET_{AX} receptor has a typically ET_A type binding profile but no affinity for the ET_A receptor selective antagonist, BQ-123, therefore it may simply be the amphibian form of the mammalian ET_A receptor.

1.3.2 Endothelin receptor structure, binding and signalling

The ET_A and ET_B receptors are classical heptahelical G-protein coupled receptors (Figure 1.3). They share a structure consisting of an extracellular N-terminal region, seven helical transmembrane loops connected by hydrophilic domains, and a 60 amino acid intracellular carboxy terminal region.

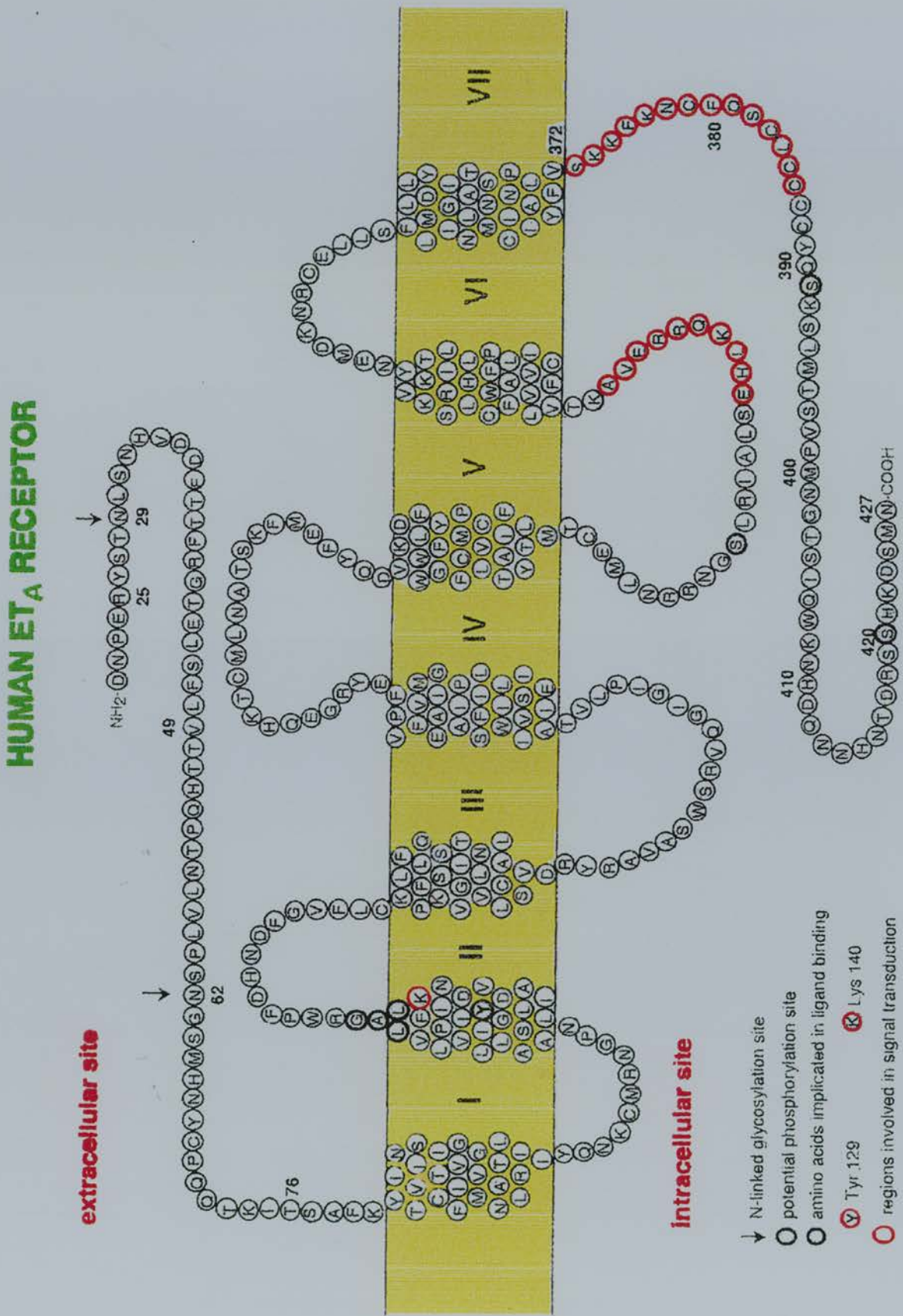
The hydrophobic transmembrane domains and the interconnecting cytoplasmic loops are highly conserved, in contrast to the N-terminal region, which shows only 4% sequence homology between the ET_A and the ET_B receptors (Elshourbagy, et al., 1993). Investigation into the structural determinants of receptor function, using site directed mutagenesis and receptor chimera techniques, has allowed identification of the sructural requirements for ligand selectivity (Adachi, et al., 1994; Sakamoto, et al., 1992).

Once bound to the receptor, a number of signal transduction mechanisms have been suggested to be involved in ET-1 mediated vascular effects. These include an early rapid rise in $[Ca^{2+}]_i$ through its release from intracellular stores and opening of membrane Ca^{2+} channels; G-protein activated phospholipase C (PLC) leading to IP3 formation and diacylglycerol (DAG) accumulation; activation of protein kinase C

(PKC); activation of phospholipase A_2 and D increasing production of arachidonic acid, and hence of prostacyclin (PGI_2) and thromboxane A_2; an increase in the intracellular pH through an effect on the sodium-hydrogen ion exchange membrane pump (full review in Rubyani & Polokoff (Rubyani and Polokoff, 1994)).

The characteristically slow onset and sustained vasoconstrictor effects described with ET-1 (Yanagisawa, et al., 1988) are thought to result from internalisation of the ligand-receptor complex and relatively slow recycling of receptors. The delayed action of ET-1 appears to result from the time required to activate the receptor-coupled second messenger systems and the prolonged duration of action may be due to continued receptor signalling following internalisation. The irreversible nature of endothelin receptor binding is most likely explained by internalisation of the receptor-ligand complex. However, binding at the cell surface receptors is more easily dissociated. Indeed, slow reversal of ET-1 mediated vasoconstriction has been demonstrated following exposure to ET receptor antagonists (Warner, et al., 1994). This may reflect reversal of binding at the cell surface receptors or result from inhibition of ET-1 binding as receptors are recycled.

Figure 1.3 The predicted structure of the human ET_A receptor with its 7 transmembrane spanning domains. Adapted from Adachi et al (1993) (Adachi, et al., 1993).



1.3.3 Endothelin receptor agonists

Understanding of the function of endothelin receptors has been aided by the use of specific pharmacological agonists and antagonists (Table 1.2). Endothelin-1 has a similar binding affinity for ET_A and ET_B receptors – in the nanomolar range – but has a much higher binding affinity for the ET_A receptor than ET-3. In contrast, ET-1 and ET-3 have equal affinity for the ET_B receptor. Currently, there are unfortunately no selective agonists at the ET_A receptor, however ET-3, SFTX6c and BQ-3020 are among a number of selective agonists at the ET_B receptor.

The endothelin and sarafotoxin peptides possess four cysteinyl residues that form two disulphide bridges, three polar charged side chains (residues 8-10) and a well-conserved hydrophobic C-terminus (residues 16-21, Figure 1.2). Examination of the binding characteristics of these peptides reveals that the ET_A receptor has much more rigid structural requirements for ligand binding than the ET_B receptor (Adachi, et al., 1994; Sakamoto, et al., 1992). Both the amino-terminal loop structure and the carboxy terminal linear portion with Trp in position 21 are vital for high affinity ET_A receptor binding. In contrast, only the linear carboxy terminal and the Trp^{21} are essential for high affinity binding to the ET_B receptor (Adachi, et al., 1994; Sakamoto, et al., 1992). ET-3 and SFTX6c are ET_B selective ligands, ET-3 having ~2000-fold and SFTX6c ~30 000-fold selectivity for binding to ET_B rather than the ET_A receptor (Williams, et al., 1991). Although both of these ligands contain loop and linear portions like ET-1, they have different amino acid sequences within the inner loop portion, which might account for their lower affinity at the ET_A receptor. BQ-3020 (Ihara, et al., 1992) is a linear analogue of ET-1 which acts as an ET_B receptor agonist, confirming that the loop portion is less important for ET_B receptor selectivity.

Table 1.2 Endothelin receptor agonists and antagonists.

			Potency (IC_{50}, K_i*)		
		selectivity	**ET_A**	**ET_B**	**Reference**
Agonist	**ET-1**	**ET_A/ET_B**	160 pM	110 pM	(Saeki, et al., 1991)
	ET-3	**ET_B**	140 nM*	64 pM*	(Williams, et al., 1991)
	SFTX6c	**ET_B**	>7300 nM*	0.25 nM*	(Williams, et al., 1991)
	BQ-3020	**ET_B**	330 nM	320 pM	(Saeki, et al., 1991)
Antagonist	**BQ-123**	**ET_A**	7.3 nM	18 μM	(Ihara, et al., 1991)
	BQ-788	**ET_B**	1.3 μM	1.2 nM	(Ishikawa, et al., 1994)
	TAK-044	**ET_A/ET_B**	0.1 nM	1.8 nM	(Kikuchi, et al., 1994)
	Bosentan	**ET_A/ET_B**	4.7 nM	95 nM	(Clozel, et al., 1994)
	BMS 193884	**ET_A**	1.4 nM*	18.8 μM*	Unpublished data[1]
	L-753,037	**ET_A/ET_B**	0.12 nM*	0.17 nM*	(Zhao, et al., 1999)

1.3.4 Endothelin receptor antagonists

Since the first description of compounds that could inhibit the binding or actions of ET-1 in 1991 (Ihara, et al., 1991; Spinella, et al., 1991), a large number of endothelin receptor antagonists, peptide and nonpeptide, selective and nonselective, have become available (Table 1.2) (Warner, et al., 1994). BQ-123 (Ihara, et al., 1991) and BQ-788 (Ishikawa, et al., 1994) are selective peptide antagonists at ET_A and ET_B receptors respectively. Non-selective antagonists include bosentan (Clozel, et al., 1994) and TAK-044 (Watanabe, et al., 1995). However, non-selective antagonists may have some degree of selectivity for the ET_A receptor, with few antagonists truly balanced at both receptors. Indeed, TAK-044 (Kikuchi, et al., 1994) and bosentan (Clozel, et al., 1994) are both described as non-selective receptor antagonists but have a relative degree of selectivity for the ET_A receptor with ~20-fold higher affinity for the ET_A receptor than the ET_B receptor.

Peptide antagonists have been obtained by chemical modification of ET-1 itself, or of microbial products with endothelin receptor binding activity (Ihara, et al., 1991; Spinella, et al., 1991). BQ-123 is a cyclic pentapeptide derived from microbial broth that has relatively high potency for binding to the ET_A receptor with approximately 2000 fold affinity for the ET_A receptor than the ET_B receptor (Ihara, et al., 1992). BQ-788 is a peptide ET_B receptor antagonist with 1000 fold selectivity for the ET_B receptor in human cell lines (Ishikawa, et al., 1994). Both of these compounds have provided valuable pharmacological tools in the investigation of endothelin physiology and pathophysiology.

Although useful as research tools, the potential of peptides as therapeutic agents may be limited by their short duration of action, as well as by their lack of oral availability. Potent nonpeptide antagonists have been developed through optimisation of compounds isolated from plant extracts (Fujimoto, et al., 1992), and microbial broths

(Ohashi, et al., 1992) or screened from chemical libraries (Clozel, et al., 1993; Clozel, et al., 1994). These compounds can be administered orally, which is of obvious advantage in the treatment of chronic disease.

1.3.5 Distribution of the ET_A and ET_B receptors

ET_A receptor mRNA has been detected in many tissues, with the highest expression in aorta, heart and kidney. The ET_A receptor predominates on vascular smooth muscle cells and is responsible for causing vasoconstriction in both large and small blood vessels (Davenport and Maguire, 1994). It is also the major receptor subtype in the heart (Molenaar, et al., 1993) and renal arterioles, bronchial smooth muscle and glandular tissues have been shown to preferentially express ET_A receptors (Arai, et al., 1990). In contrast, ET_A mRNA cannot be detected in the liver or endothelial cells (Hosada, et al., 1991).

ET_B receptor mRNA is most abundant in endothelial cells (Hosada, et al., 1991; Molenaar, et al., 1993; Ogawa, et al., 1991) and can also be detected in vascular smooth muscle cells (Davenport, et al., 1993) and is predominantly found in brain, lung, kidney and aorta.

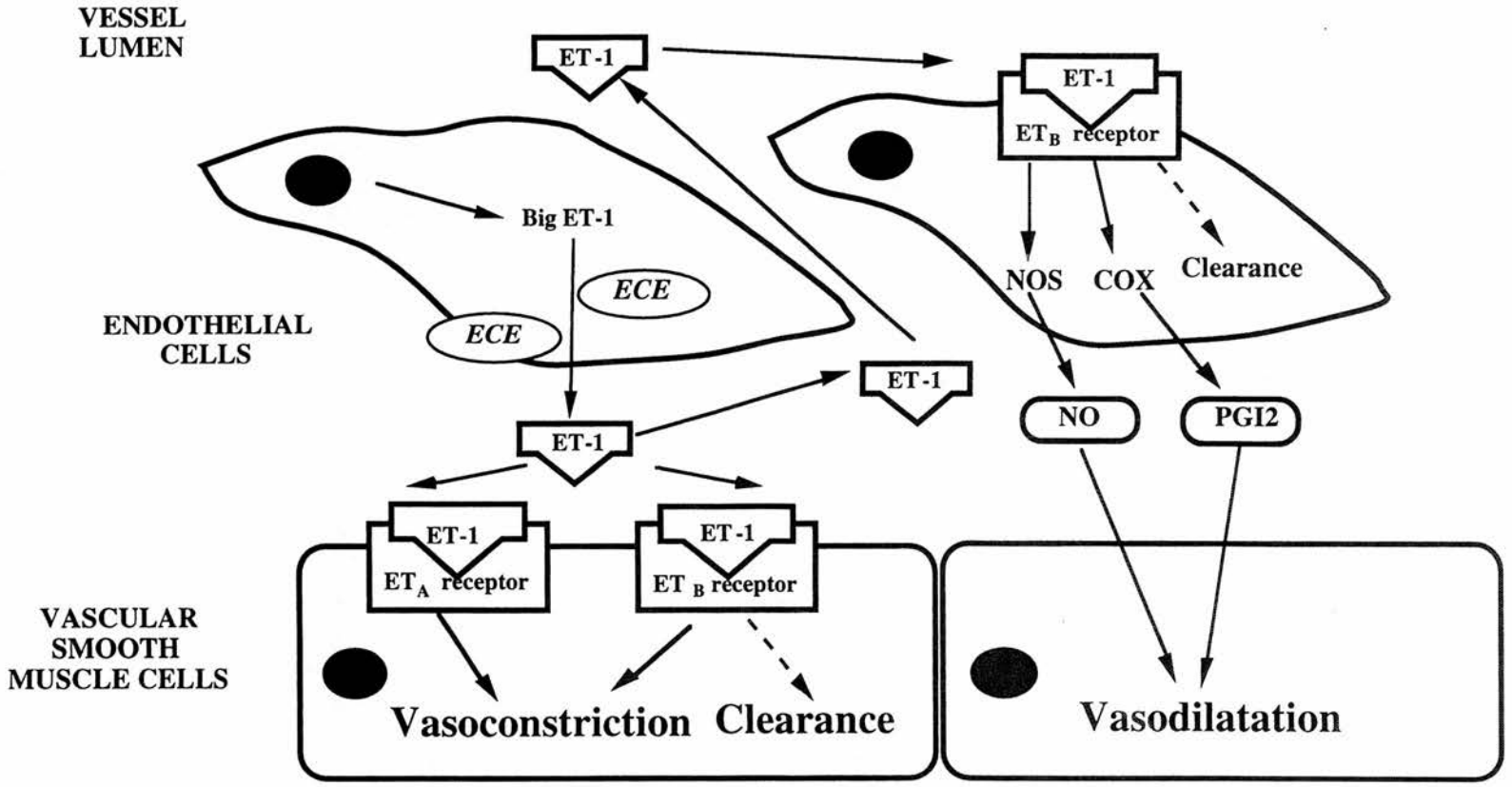
1.4 Cardiovascular effects of Endothelin

Endothelin-1 is an important mediator of vascular tone (Haynes and Webb, 1994). Indeed, ET-1 is a more potent vasoconstrictor and pressor agent even than angiotensin II (Yanagisawa, et al., 1988). In addition to its direct vasoconstrictor actions, ET-1 can potentiate the vasoconstrictor effects of other constrictor substances including noradrenaline and angiotensin II (Yang, et al., 1990). Endothelin-1 has co-mitogenic effects (Wang, et al., 1994) and, so may play a role in pathophysiological vascular and cardiac remodelling and cell proliferation. The direct and indirect effects of ET-1 on vascular function indicate its potential importance in the cardiovascular physiology.

1.4.1 Endothelin and vascular tone

In vascular tissue, ET_A receptor mRNA is expressed predominantly in smooth muscle (Arai, et al., 1990; Hori, et al., 1992), while ET_B receptor mRNA is most abundant in endothelial cells (Hosada, et al., 1991; Molenaar, et al., 1993; Ogawa, et al., 1991). These findings are consistent with the view that ET-1 constriction of vascular smooth muscle is mediated predominantly by ET_A receptors and that constriction is modified by release of relaxing factors from the endothelium, including prostacyclin (DeNucci, et al., 1988) and nitric oxide (Tsukahara, et al., 1994), through stimulation of ET_B receptors (Figure 1.4). However, ET_B receptor mRNA is detectable in vascular smooth muscle cells (Davenport, et al., 1993) and ET_B receptor selective agonists can mediate constriction *in vitro* and pressor responses *in vivo* (Clozel, et al., 1992; Gray, et al., 1994; Moreland, et al., 1992; Seo, et al., 1994). In addition, although ET_A receptor selective antagonists attenuate ET-1 mediated vasoconstriction, the response is not completely abolished (Gardiner, et al., 1994; McMurdo, et al., 1993; Seo, et al., 1994). These observations suggest the presence of ET_B receptors that mediate constriction of vascular smooth muscle cells (Figure 1.3). However, the physiological relevance of this constrictor response remains to be confirmed. The contribution of ET_B receptors to ET-1 mediated vasoconstriction is variable, with *in vitro* studies reporting that ET_B receptors make either a minimal (Davenport and Maguire, 1994; Fukuroda, et al., 1994; Godfraind, 1993; Maguire, et al., 1994; Riezebos, et al., 1994) or, at most, a moderate contribution (Seo, et al., 1994; White, et al., 1994) to vasoconstriction. Responses appear to depend markedly on species, vessel type and vessel size (Davenport and Maguire, 1994).

Figure 1.4 Endothelin-1 is generated by endothelial cells and is secreted abluminally to act on both ET_A and ET_B receptors. ET_A mediates the potent vasoconstrictor effects of endothelin-1. The ET_B receptor mediates vasodilatation through generation of nitric oxide and prostacyclin. The ET_B receptor may also mediate vasoconstriction and acts as a clearance receptor for endothelin-1.



The ET_B receptor is also thought to be involved in clearance of endothelin from the circulation (Fukuroda, et al., 1994). In support of this, plasma concentrations of ET-1 are dose dependently increased following administration of combined ET_A/ET_B (Loffler, et al., 1991) and selective ET_B (Fukuroda, et al., 1994) receptor antagonists but not following administration of selective ET_A receptor antagonists. It is possible that the vasoconstrictor effects of selective ET_B receptor antagonism result partly from inhibition of ET_B mediated clearance of ET-1, allowing the uncleared peptide to bind to unoccupied constrictor ET_A receptors.

The balance between the constrictor effects of ET-1, mediated by the vascular smooth muscle ET_A and ET_B receptors, and the dilator effects mediated by the endothelial ET_B receptor is important in determining the value of combined ET_A/ET_B and selective endothelin receptor antagonists. There may be alterations in endothelin receptor function, distribution and sensitivity in disease states, which could lead to an imbalance between the actions of ET-1 on the ET_A and ET_B receptors. For example, ET_B receptor expression is increased during the change of cultured vascular smooth muscle cells from a contractile to synthetic phenotype (Eguchi, et al., 1994), in hypertension (Batra, et al., 1993) and under the influence of angiotensin II (Kanno, et al., 1993).

The importance of ET-1 in the maintenance of vascular tone in healthy blood vessels has been highlighted in recent clinical studies, which have confirmed the constrictor effects of ET-1 (Clarke, et al., 1989) and the vasodilator effects of ET_A receptor antagonism (Haynes and Webb, 1994) *in vivo* . Further investigation is now required to identify the relative contribution of the endothelin receptor subtypes to the vascular effects of ET-1.

1.4.2 Endothelin and blood pressure

In the original description of ET-1 (Yanagisawa, et al., 1988), a sustained increase in blood pressure was demonstrated following intravenous administration of a bolus dose of ET-1 in denervated rats. Similar pressor responses have been demonstrated by other investigators in other species. ET-2 and ET-3 have also been shown to increase blood pressure although to a lesser degree than ET-1. The increase in blood pressure following continuous infusion of ET-1 is thought to be mediated through an increase in peripheral vascular resistance (Mortensen, et al., 1990) and has been prevented by salt restriction (Mortensen and Fink, 1992). This could reflect increased vascular sensitivity to ET-1 with a high salt diet or an interaction with the renin-angiotensin system.

The sustained increase in arterial pressure occurs despite the rapid clearance of ET-1 from the circulation (Anggard, et al., 1989), consistent with slow dissociation of ET-1 from its receptors (Hirata, et al., 1988). Interestingly, both ETB receptor selective agonists and ET-1 increase blood pressure in animal models (Clozel, et al., 1992; Yanagisawa, et al., 1988). Some investigators have shown that the pressor response to ET-1 is not completely blocked by ET_A receptor selective antagonists (Gardiner, et al., 1994) but can be blocked following administration of the non-selective ET receptor antagonist bosentan (Gardiner, et al., 1994), indicating the potential contribution of the ETB receptor in this pressor response. Intravenous infusion of ET-1 has also been shown to increase blood pressure in humans (Vierhapper, et al., 1990) which is not affected by nifedipine, a Ca^{2+} channel blocker or by indomethacin, a cyclo-oxygenase inhibitor (Vierhapper, et al., 1992).

Transient hypotension, preceding the sustained pressor effects, has been noted following bolus doses of the endothelin peptides. This effect is most noticable with ET-3 (Inoue, et al., 1989) and is thought to be caused by generation of endothelium-

derived dilators nitric oxide and prostacyclin, mediated by endothelial cell ET_B receptors. This depressor response has been attenuated by inhibition of nitric oxide synthase (Fozard and Part, 1992) and cyclo-oxygenase (Filep, et al., 1993). However, the effects of cyclo-oxygenase inhibition described are not universal (DeNucci, et al., 1988) and although likely to be mediated by the actions of the endothelial ET_B receptor, no single mediator of this transient depressor effect has been proposed. Given the potent and well recognised constrictor effects of ET-1, this dilator response is more likely to represent a pharmacological rather than a physiological response. However, it provides important evidence of the dilator effects of endothelial ET_B receptor stimulation.

1.4.3 Cardiac effects

Synthesis of ET-1 has been detected in rat (Suzuki, et al., 1993), porcine (Tonnessen, et al., 1995) and human cardiomyocytes (Giaid, et al., 1995) and both ET_A and ET_B receptor subtypes are present on cardiomyocytes (Molenaar, et al., 1993; Ono, et al., 1995). Antagonist studies have shown an alteration in coronary flow of isolated rat hearts (Wang, et al., 1994), suggesting that ET_A and ET_B receptors are present in the coronary vasculature. Indeed marked coronary vasoconstriction has been described following administration of ET-1 in animal models (Clozel and Clozel, 1989; Pernow, et al., 1989). The initial hypotension produced by ET-1 is associated with an increase in heart rate and cardiac output, suggesting that it is secondary to systemic vasodilatation. In contrast, the pressor response to ET-1 is associated with bradycardia and a reduction in stroke volume, causing a marked reduction in cardiac index (Miller, et al., 1989). In addition, ET-1 tends to reduce cardiac output (Wagner, et al., 1992) and coronary blood flow (Pernow, et al., 1996). In addition to these vasoconstrictor effects, both anti- and pro-arrhythmic effects of ET-1 have been described and, in a rat acute ischaemia model, the ET_A receptor selective antagonist BQ-123 has been shown to be anti-arrhythmic at low doses but pro-arrhythmic at higher, and possibly non-

selective, doses (Garjani, et al., 1995). Further investigation of the arrhythmic effects of ET-1 is required.

1.4.4 Renal effects

ET-1 has two main direct actions on the kidney, renal vasoconstriction and increased tubular sodium and water loss. These effects probably reflect separately regulated ET systems in the renal vasculature and renal tubules. Indeed, in the human kidney, the ET_B receptor predominates over the ET_A receptor, with the ET_A receptor largely confined to the vasculature and the ET_B receptor more abundantly expressed in the mesangial cells (Karet, et al., 1993). In contrast, the ET_A receptor predominates in the rat kidney with renal vasoconstriction mediated mainly by ET_B receptors (Clozel, et al., 1992).

Administration of low dosages of ET-1 in humans produces sustained renal vasoconstriction (Gasic, et al., 1992; Rabelink, et al., 1994; Weitzberg, et al., 1991) and induces sodium retention (Rabelink, et al., 1994). In contrast, studies in animal models have consistently shown a natriuretic effect of ET-1 adminstration (Garcia, et al., 1990; Perico, et al., 1991), despite a fall in GFR and renal blood flow. These findings suggest that the actions of endothelin on sodium excretion depend on a balance between sodium retaining and natriuretic factors. In humans, it appears that the predominant effect of small increases in plasma endothelin concentration is sodium retention (Rabelink, et al., 1994). Given these inter-species variations, caution should be taken when extrapolating the results from animal experiments to humans, further clinical investigation of the renal effects of ET receptor antagonists is required.

1.5 The role of endothelin in cardiovascular disease

As previously discussed, ET-1 plays an important role in the regulation of vascular tone and blood pressure *in vivo* (Haynes, et al., 1996) due to its powerful and

sustained vasoconstrictor effects (Clarke, et al., 1989). ET-1 has been implicated as a mediator of the raised peripheral vascular resistance associated with a number of cardiovascular diseases, including essential (or primary) hypertension, chronic heart failure and chronic renal failure. In addition to its direct vasoconstrictor actions, ET-1 can potentiate the effects of other vasoconstrictor substances including noradrenaline and angiotensin II (Yang, et al., 1990). Endothelin-1 has co-mitogenic effects (Wang, et al., 1994) and, so may play a role in vascular modelling and cell proliferation. Plasma concentrations of ET-1 are increased in a number of cardiovascular diseases including, heart failure (Pacher, et al., 1993; Wei, et al., 1994), renal failure (Koyama, et al., 1989; Warrens, et al., 1990), and myocardial infarction (Omland, et al., 1994) and, in most cases, the increase in plasma ET concentrations correlates with markers of disease progression. Although it is unclear whether the increase in plasma concentrations of ET-1 is merely a marker of disease progression, the direct and indirect effects of ET-1 on vascular function support a functional role for ET-1 in the pathophysiology of a number of cardiovascular diseases.

1.5.1 Heart failure

Plasma concentrations of ET-1 and its precursor, big ET-1, are elevated in patients with heart failure and correlate with mortality and with the need for cardiac transplantation (Pacher, et al., 1993; Wei, et al., 1994). Although the increase in plasma ET-1 may result from reduced renal clearance, the increase in big ET-1 concentrations suggests an increase in ET-1 generation. Given the potent vascular effects of ET-1, the correlation between measures of mortality and morbidity and the increase in plasma levels of ET-1 and big ET-1, the endothelin system is likely to be of functional importance in the pathophysiology of heart failure. Early clinical studies have demonstrated benefits of endothelin antagonists in the presence and absence of existing treatment with an ACE inhibitor. Systemic administration of the non-selective ET receptor antagonist bosentan in patients with heart failure produced significant

systemic and pulmonary vasodilatation; reducing mean arterial pressure, pulmonary arterial pressure, right atrial pressure and pulmonary artery wedged pressure (Kiowski, et al., 1995). Cardiac index was also increased with no change in heart rate. Further investigation of the systemic effects of combined ET_A/ET_B and selective endothelin receptor antagonism in heart failure is required to confirm which approach will be of more benefit in the clinical setting. Phase II and III trials investigating selective and combined ET_A/ET_B endothelin antagonists in the treatment of heart failure are currently in progress.

Given the increase in plasma concentrations of big ET-1 in heart failure (Pacher, et al., 1993; Wei, et al., 1994) and the proposal that this indicates an increase in the generation of ET-1, ECE inhibitors may also be of value in treating chronic heart failure.

1.5.2 Hypertension

Plasma endothelin concentrations are not normally raised in essential hypertension but have been described in patients with coexisting renal disease (Koyama, et al., 1989). Nevertheless, the potent vasoconstrictor and vasopressor actions of ET-1 may play a role in the development of increased blood pressure and in the pathophysiology of disease progression. The co-mitogenic properties of ET-1 may be important in the hypertrophic and atherosclerotic process, again associated with an increasing cardiovascular risk. Early studies with endothelin receptor antagonists in hypertension have been promising. Local vasodilatation has been demonstrated in response to the ET_A selective antagonist BQ-123 (Ihara, et al., 1992) in healthy volunteers (Haynes and Webb, 1994). Interestingly, a single oral dose of the combined ET_A/ET_B antagonist bosentan was effective in lowering blood pressure in patients with essential hypertension (Schmitt, et al., 1995). In essential hypertension, endothelin antagonists may provide additional blood pressure reduction in combination with existing therapies

or may be useful in treating hypertension which has proven resistant to current methods of treatment. The combined vasoconstrictor and co-mitogenic properties of ET-1 provide an argument for the use of endothelin antagonists in this condition and the results of further studies are awaited.

1.5.3 Chronic renal failure

Plasma concentrations of ET-1 are elevated in renal disease (Koyama, et al., 1989; Warrens, et al., 1990) and are likely to result from a reduced renal clearance of ET-1 rather than increased production of the peptide. However, it is also likely that ET-1 is involved in the progression of renal disease (Benigni, 1995). Indeed, in a rat model of progressive renal disease, renal ET-1 gene expression correlated with disease progression (Orisio, et al., 1993). From animal studies, there is increasing evidence that ET-1 plays a role in the progression of renal disease. Investigation of the role of ET-1 and the endothelin receptor subtypes in renal function and the progression of renal disease in humans is ongoing.

Although the ET_B receptor subtype predominates in the human kidney (Karet, et al., 1993), a greater proportion of ET_A receptors are localised to the vasculature and the ET_A subtype is likely to be important in mediating renal vasoconstriction to ET-1 (Davenport, et al., 1994). The selective ET_A receptor antagonist FR139317 appeared to be successful in preventing disease progression in a rat model of chronic renal disease by reducing proteinuria, markers of cell proliferation and providing evidence of protection against glomerular structural injury (Benigni, et al., 1993). It is possible that tubular ET_B receptors offer renoprotection in ischaemic conditions through sodium and water excretion. Theoretically, it would therefore be appropriate to block the ET_A receptor selectively and preserve ET_B mediated diuresis and natriuresis. However, it is important to interpret the results of studies in animal models with caution, due to species differences in renal physiology (Becker, et al., 1994) and in endothelin

receptor distribution. Early clinical trials with endothelin antagonists are being conducted in patients with chronic renal failure. The results of such studies will help identify the potential of endothelin antagonists in a clinical context.

1.5.4 Acute renal failure

Acute renal failure develops over a period of hours to days and can result from tubular ischaemia, obstruction or toxic insult, or from a marked reduction in renal perfusion. It is associated with a high mortality and is a recognised complication of cardiac surgery and administration of radio-opaque contrast agents. Renal vasoconstriction is thought to be important in the pathophysiology of radiocontrast nephropathy, and this may be mediated by ET-1 (Cantley, et al., 1993). Indeed, plasma endothelin concentrations have been shown to increase following intravascular administration of radiocontrast agent in rats (Heyman, et al., 1992). The potent vasoconstrictor effects of ET-1, combined with its effects on renal blood flow and glomerular filtration, suggest a role for endothelin in the pathophysiology of ischaemic acute renal failure (Clozel, et al., 1993; Pollock and Opgenorth, 1994). In addition to the beneficial effects described in chronic renal failure, selective and combined ET_A/ET_B endothelin antagonists have also been shown to limit disease progression in acute models of renal failure (Benigni, 1995; Brooks, et al., 1994; Clozel, et al., 1993; Mino, et al., 1992). Interestingly, administration of the ECE/NEP inhibitor phosphoramidon (Ikegawa, et al., 1991) was more effective in restoring renal function and in minimising structural changes than selective ET_A antagonism with BMS-182874 in an animal model of ischaemic renal failure (Bird, et al., 1995). Again, further studies and direct comparison of selective and combined ET_A/ET_B antagonists are required to confirm the potential of these compounds in the clinical setting.

1.5.5 Myocardial infarction

Plasma ET-1 concentrations are increased following myocardial infarction and are strongly related to mortality (Omland, et al., 1994). The vascular effects of ET-1 indicate a potential role in the progression of myocardial damage to heart failure. However, it is not clear whether ET-1 plays a role in infarct development. The potential of endothelin antagonists in the treatment of myocardial infarction and the prevention of the progression of cardiovascular disease remain to be confirmed.

1.5.6 Subarachnoid haemorrhage

Subarachnoid haemorrhage carries a risk of rebleeding and of cerebral ischaemia associated with cerebral vasospasm. Although the mechanism involved in cerebral vasospasm is unclear, ET-1 is a likely mediator of this effect (Roux, et al., 1995). In support of this, combined ET_A/ET_B antagonism with bosentan was effective in reversing (Roux, et al., 1995) cerebral vasospasm in a rabbit model of subarachnoid haemorrhage. Selective ET_A receptor antagonism, with BQ-485 (Itoh, et al., 1993) is also effective in limiting cerebral vasospasm in a dog model of subarachnoid haemorrhage. Endothelin-1 may also be involved in the development of cerebral ischaemia through cerebral vasoconstriction. Further investigation of selective and combined ET_A/ET_B endothelin antagonists in the treatment of subarachnoid haemorrhage will reveal the true potential of these compounds in this context.

1.5.7 Potential therapeutic benefits of endothelin receptor antagonists and endothelin converting enzyme inhibitors

The therapeutic potential of a number of endothelin receptor antagonists is currently under investigation in phase II and phase III clinical trials in heart failure and in hypertension. Demonstration of a vasodilator response to these compounds, especially in the presence of existing therapies, is vital if endothelin antagonists are to be indicated as complimentary treatments in cardiovascular disease. Orally active

compounds are of particular importance in the treatment of chronic disease. Once initial benefits have been demonstrated, long term trials will be necessary to confirm their benefits in terms of prevention of disease progression. Phase II and III clinical trials investigating the long term administration of orally active compounds in the treatment of patients with heart failure are in progress. Intravenous compounds are currently being investigated as potential treatments in acute renal failure. Studies in animal models have proven promising and clinical trials are now required to confirm the potential of these compounds.

The question of whether combined ET_A/ET_B or selective receptor antagonism will be of more benefit as vasodilator treatments depends on the balance between the ET_B receptor mediated effects of vasodilatation, vasoconstriction and clearance. Further investigation of the actions mediated by the ET_B receptor in health and in specific disease states is necessary to answer this question. This issue is of particular relevance in cardiovascular diseases where there is associated endothelial dysfunction and there may be a reduced capacity for ET_B receptor mediated dilatation.

ECE inhibition may be a useful approach in limiting the vascular effects of ET-1 in conditions where its generation is increased. ECE inhibitors are likely to have systemic vasodilator effects by directly inhibiting the generation of ET-1 and indirectly by removal of the inhibitory effects of ET-1 on nitric oxide generation (Boulanger and Luscher, 1990). Indeed, local vasodilatation was demonstrated following local administration of the non-selective ECE/NEP inhibitor, phosphoramidon in humans *in vivo* (Haynes and Webb, 1994). However, the development of ECE-inhibitors as potential therapeutic agents is at least several years behind the development of endothelin receptor antagonists. Furthermore, even if selective and potent ECE-inhibitors were to be discovered, they would still have to overcome the problem of

accessability should the intracellular ECE prove to be of more importance physiologically.

Although much of the research interest is focused on the potential of endothelin receptor antagonists in cardiovascular disease, endothelin receptor antagonists may also be of value in the treatment of a number of conditions as diverse as bronchospasm (Fukuroda, et al., 1996), prostate cancer (Nelson, et al., 1996), portal hypertension (Gunal, et al., 1996), and gram positive infection (Uosaki, et al., 1996).

1.6 Aims and Hypotheses

Vasoconstriction to ET-1 has previously been demonstrated in a number of animal models and in humans *in vivo*. Two human ET receptors have been described, the ET_A and the ET_B receptors. Endothelin receptor agonists and antagonists have subsequently been described and provide valuable pharmacological tools in the investigation of the endothelin system. The constrictor effects of ET-1 are largely mediated by the ET_A receptor. However, the contribution of the ET_B receptor to ET-1 mediated vasoconstriction and the overall balance of effects at the endothelial cell and vascular smooth muscle cell ET_B receptors remain to be confirmed.

In a series of clinical studies in healthy volunteers, the following hypotheses will be addressed:

- ET_B receptor stimulation causes vasoconstriction in resistance and capacitance vessels (Chapter 3, 4, and 5);
- the ET_B receptor contributes to ET-1 mediated vascular tone (Chapter 4);
- the vasoconstrictor and vasodilator effects of ET_B receptor stimulation are modulated by the endothelium-derived dilators, nitric oxide and prostacyclin (Chapter 4 and 5);

- ET_B receptor antagonism will affect the response to selective ET_A receptor blockade and will demonstrate the endogenous effects of the ET_B receptor (Chapter 6).
- intra-arterial infusion of ET-1 at locally active doses, with assessment of local forearm vasoconstriction by venous occlusion plethysmography, provides a reproducible and valuable model for proof of concept studies with endothelin receptor antagonists (Chapter 7);
- selective ET_A and non-selective ET_A/ET_B receptor antagonists will block the effects of exogenously administered ET-1 and provide evidence of blockade of the endogenous effects of ET-1 (Chapter 8 and 9);
- systemic ET_B receptor antagonism will demonstrate the endogenous effects of the ET_B receptor in the maintenance of vascular tone (Chapter 10).

These hypotheses will be addressed by assessment of the effects of endothelin receptor agonists and antagonists on local and systemic measures of vascular tone.

Chapter 2

Methods

2. METHODS

As a first step in understanding the mechanisms involved in the role of the endothelin system in the maintenance of vascular tone *in vivo,* the initial studies involved the assessment of the effects of locally active doses of vasoactive agents on local measures of vascular tone. The vasoactive compounds were infused either intra-venously, to assess effects in capacitance vessels, or intra-arterially, to assess effects in the resistance vessels. The use of locally active, subsystemic doses allows assessment of the direct vascular effects of these compounds in intact vessels exposed to normal physiological conditions (Webb, 1995), without the confounding effects on other organs such as the brain, heart and kidneys and consequent neurohumoral reflexes, associated with systemic drug administration.

In subsequent studies, the effects of systemically active doses of endothelin receptor antagonists were assessed alone and in combination with local infusions, to further investigate the role of endothelin receptor subtypes in the maintenance of vascular tone *in vivo.*

All studies were conducted with the approval of the Lothian Medicine and Clinical Oncology Ethics of Medical Research Sub-Committee and the written informed consent of each subject. The investigations conformed with the principles outlined in the Declaration of Helsinki. No subject received vasoactive or non-steroidal anti-inflammatory drugs in the week before each phase of the study, and all abstained from alcohol for 24 hours, and from food, caffeine containing drinks and cigarettes for at least 3 hours before any measurements were made. All studies were performed in a quiet room maintained at a constant temperature between 23 and 25°C.

2.1 Drug administration

2.1.1 Locally active doses - intra-venous administration

A vein on the dorsum of the hand of the non-dominant arm was cannulated in the direction of flow with a 23 SWG butterfly needle (Abbott, Sligo, Republic of Ireland) attached to a 16G epidural catheter, without use of local anaesthesia (Figure 2.1). The same vein was used in each subject for each individual study. Patency was maintained by infusion of 0.9% physiologic saline via a syringe pump (Welmed P1000: Welmed Clinical Care Systems, Bramley, Hampshire, UK; Chapter 3, Study 1 or IVAC P1000: IVAC Ltd, Basingstoke UK; all other studies). In each study, saline was infused for 30 min prior to the infusion of the study agent. The total rate of infusion was maintained constant throughout all studies at 0.25 ml/min.

2.1.2 Locally active doses - intra-arterial administration

The brachial artery of the non-dominant arm was cannulated under local anaesthesia (1% lignocaine; Astra Pharmaceuticals, Kings Langley, UK) with a 27 SWG steel needle (Coopers Needle Works, Birmingham, UK) attached to a 16G epidural catheter (Portex Ltd, Hythe, Kent, UK) (Figure 2.2). Patency was maintained by infusion of 0.9% physiologic saline via a syringe pump (IVAC P1000). In each study, saline was infused for 30 min prior to the infusion of the study agent. The total rate of intra-arterial infusion was maintained constant throughout all intra-arterial studies at 1 ml/min.

Cannulation sites were examined for any adverse effects following studies and volunteers were contacted 24 hours after the study to monitor for any symptoms resulting from the cannulation procedure.

Figure 2.1 A selected dorsal hand vein cannulated with a 23 SWG butterfly needle for local intra-venous infusion of study compounds. The diameter of the vein is measured by displacement of a magnetic rod supported within the linear variable differential transformer by means of a tripod.

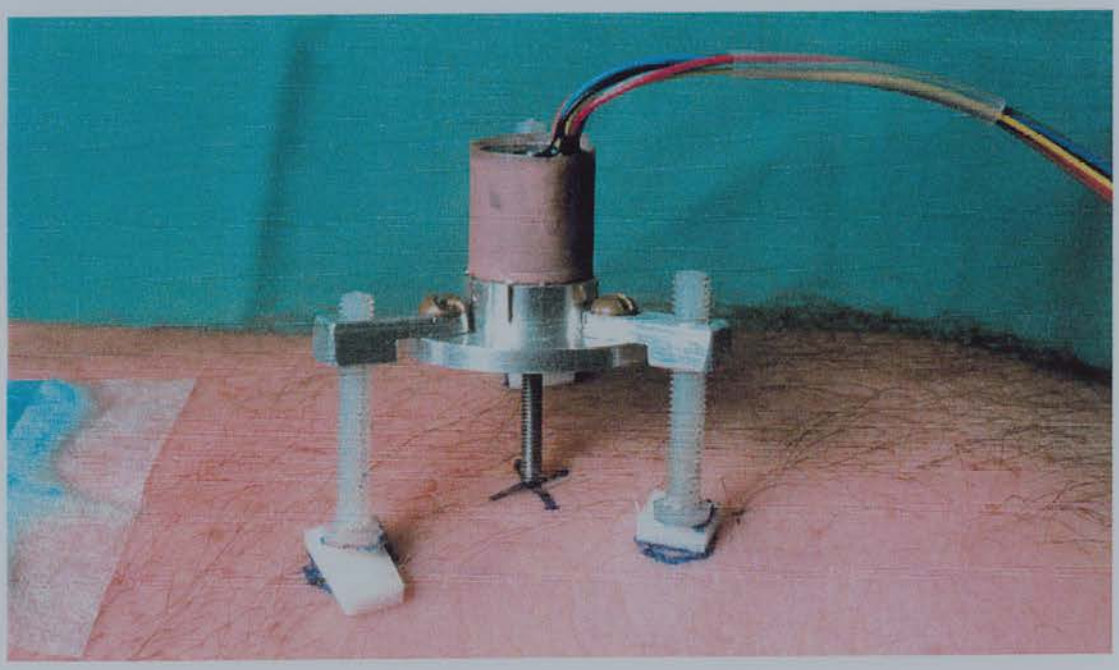


Figure 2.2 The brachial artery of the non-dominant arm cannulated with a 27 SWG steel cannula for local intra-arterial infusion of study compounds.

2.1.3 Systemically active doses - intravenous administration

In Chapter 8 and Chapter 10, study drugs were infused intravenously, at systemically active doses, via an 18 SWG cannula sited in an antecubital vein, at a constant rate for 60 minutes (Chapter 8) and 15 minutes (Chapter 10).

2.2 Drugs

A single dose of each endothelin receptor agonist or antagonist was used in individual studies because the slow onset and long lasting action of the endothelin isopeptides precludes the use of repeated doses in a single study to examine conventional dose-response relationships (Clarke, et al., 1989). Exact concentrations of drug are not easily obtainable in this setting. However, approximate estimation of concentrations can be made, based on a flow of ~5 ml/min in the capacitance vessels and ~50 ml/min in the resistance vessels.

All dilutions, with the exception of L-753,037 (Section 2.2.8 and Chapter 8), were prepared in 0.9% saline (Baxter Healthcare Ltd) from sterile stock solutions on the day of the study. L-753,037 was prepared under standard aseptic conditions within the hospital pharmacy department.

2.2.1 Endothelin-1

Endothelin-1 (Clinalfa, NovaBiochem, Nottingham, UK) was administered as a non-selective ET receptor agonist and infused at a locally active dose of 5 pmol/min via the dorsal hand vein (Chapters 3 & 4) and brachial artery (Chapters 7, 8 & 9) as described above. The choice of this dose was based on previous work showing, *in vivo*, that ET-1 at 5 pmol/min causes slow onset venoconstriction of ~60% in human skin capacitance vessels (Haynes and Webb, 1993) and ~40% in human resistance vessels (Haynes and Webb, 1994).

2.2.2 Sarafotoxin S6c

As discussed in Chapter 1, sarafotoxin S6c (SFTX6c) is an ETB receptor selective agonist with 30,000 fold selectivity for the ET_B receptor over the ET_A receptor (Williams, et al., 1991). Sarafotoxin S6c (Sigma Chemical Co Ltd, Nottingham, UK: Chapter 3, Study 1; Chapter 4, Study 3 & 4, and Calbiochem-Novabiochem, Nottingham, UK: Chapter 3, Study 2 & 3; Chapter 5, Study 1 & 2) was administered at locally active doses via the dorsal hand vein (Chapter 3 and Chapter 4) and the brachial artery (Chapter 5) as described above.

Sarafotoxin S6c has affinity for the ET_B receptor in the nanomolar range (K_iET_B 0.25±0.07nM, K_iET_A >7300nM; human hippocampus (Williams, et al., 1991)). Although it is not possible to quantify exact tissue concentrations during local infusion, the doses of SFTX6c used in studies in Chapter 3, Chapter 4, and Chapter 5 are estimated to be within a range selective for the ETB receptor (Table 2.1).

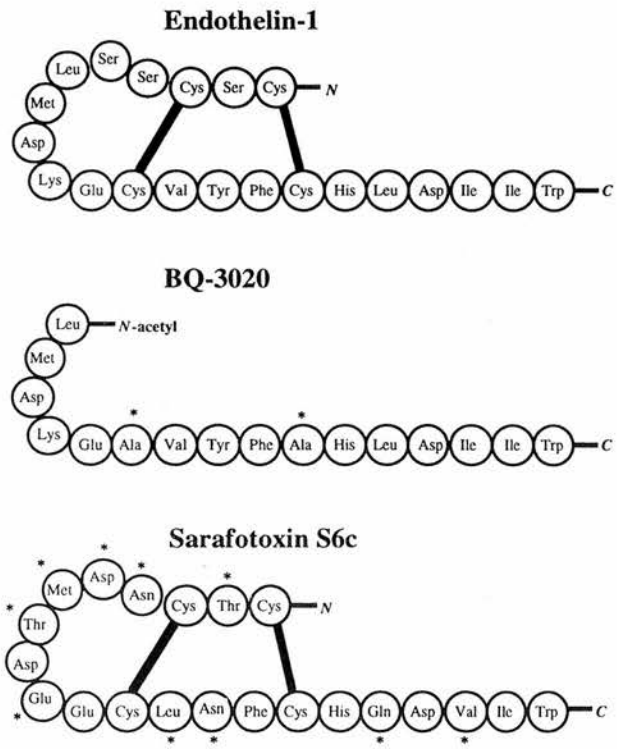
Table 2.1 Estimated tissue concentrations during infusion of SFTX6c in the doses administered in Chapter 3, Chapter 4, and Chapter 5.

SFTX6c infusion rate	**Estimated tissue concentration**			
	Vein	***Artery***	***Chapter***	***Study***
5 pmol/min	1 nM		3	1, 2, 3
			4	3, 4
		0.1 nM	5	1
50 pmol/min	10 nM		3	2
60 pmol/min		1.2 nM	5	2

2.2.3 BQ-3020

BQ-3020 is an ET_B receptor selective agonist with 4700 fold selectivity for the ET_B receptor over the ET_A receptor (Ihara, et al., 1992). It is a linear analogue of ET-1 and differs from ET-1 by 2 amino acids, over a limited stretch of peptide (Figure 2.3). BQ-3020 has a closer structural similarity to ET-1 than SFTX6c, therefore, it may be more representative of endogenous ET-1 binding than SFTX6c.

Figure 2.3 Diagramatic representation of the structures of endothelin-1, BQ-3020 and sarafotoxin S6c; asterisks denote positions of amino acids in BQ-3020 and sarafotoxin S6c which differ from endothelin-1.



* amino acids which differ from ET-1

BQ-3020 (Calbiochem-Novabiochem, Nottingham, UK) was administered by continuous infusion for 90 min (Chapter 3, Study 3; Chapter 5, Study 1) at an infusion rate of 50 pmol/min. This dose of BQ-3020 was chosen from a dose ranging (1-50 pmol/min) pilot study, in 3 volunteers. The dose selected from the pilot study (50 pmol/min) was the lowest dose at which constrictor effects were seen in the hand veins, and was not intended to be directly comparable with the dose of SFTX6c used in Chapter 3 (Study 3) and Chapter 5 (Study 1). In human tissues BQ-3020 has affinity for the ET_B receptor in the nanomolar range (K_DET_B 1.38±0.72nM, K_DET_A 1040±210nM; human heart (Peter and Davenport, 1996)). Although it is not possible to quantify exact tissue concentrations during local infusion, this dose of BQ-3020 is within a range selective for the ET_B receptor (arteries ~1 nM, veins ~10 nM).

2.2.4 BQ-123

BQ-123 is a selective ET_A receptor antagonist with 2000 fold selectivity for the ET_A over the ET_B receptor (Ihara, et al., 1992). BQ-123 (Clinalfa AG, Laufelfingen, Switzerland) was infused in dorsal hand vein studies at doses of 0.3 nmol/min (Chapter 4, Study 1) and 1 nmol/min (Chapter 4, Study 2) to assess the contribution of the ET_A receptor to the effects of exogenously applied ET-1 and SFTX6c. In Chapter 6, BQ-123 (10 nmol/min) was infused intra-arterially, as described previously (Ferro, et al., 1996).

In human tissues BQ-123 has affinity for the ET_A receptor in the nanomolar range (K_DET_A 0.73±0.22nM, K_DET_B 24.3±2.0μM; human heart (Peter and Davenport, 1996). The doses of BQ-123 used in studies in Chapter 4 & 6 are estimated to be within a range selective for the ET_A receptor (Table 2.2).

Table 2.2 Estimated tissue concentrations during infusion of BQ-123 in the doses administered in Chapter 4, and Chapter 6.

BQ-123 infusion rate	**Estimated tissue concentration**			
	Vein	***Artery***	***Chapter***	***Study***
0.3 nmol/min	60 nM		4	1
1 nmol/min	200 nM		4	2
10 nmol/min		200 nM	6	

2.2.5 BQ-788

BQ-788 is a selective ET_B receptor antagonist, with 1000 fold selectivity for the ET_B receptor, in the nanomolar range, in human cell lines (Ishikawa, et al., 1994) and on inhibition of ET-3 binding to recombinant human ET_B receptors expressed in Chinese Hamster ovary cells, also in the nanomolar range (Reynolds, et al., 1995).

Locally active administration - Intra-venous infusion

BQ-788 (American Peptide Company, Sunnyvale, CA, USA) was administered at locally active doses via the dorsal hand vein, as described above, to assess the contribution of the ET_B receptor to the effects of exogenously applied ET-1 (Chapter 4, Study 2), and SFTX6c (Chapter 4, Study 3).

Locally active administration - Intra-arterial infusion

BQ-788 (American Peptide Company) was also administered at a dose level of 1 nmol/min via the brachial artery, as described above, to assess the contribution of the ET_B receptor to the response to bolus dose of SFTX6c (Chapter 5, Study 2) and to the response to the ET_A antagonist BQ-123 (Chapter 6). Administration of BQ-788 alone allowed assessment of the endogenous effects of the ET_B receptor in the resistance vessels (Chapter 6).

Systemically active administration - Intravenous infusion

BQ-788 (Clinalfa AG, Laufelfingen, Switzerland: Chapter 10) was administered at systemically active doses (3-300 nmol/min) intravenously, as described above. The dose range used in the Chapter 10 was selected from studies investigating the local effects of BQ-788 in the forearm circulation (Chapter 5 and Chapter 6) and from a dose ranging pilot study in which 2 volunteers were studied at each dose level (data not shown). Selected doses (1-300 nmol/min) were administered in the pilot study to identify a no effect dose and select an appropriate maximum dose for the main study.

In Chapter 10, saline (0.9%; Baxter Healthcare, Ltd) was administered as placebo.

2.2.6 L-NMMA

L-N^G-monomethyl-arginine (L-NMMA) is a specific substrate analogue that acts as a competitive inhibitor of nitric oxide synthase in humans (Vallance, et al., 1989). L-NMMA (NovaBiochem, Nottingham, UK) was administered in locally active doses in dorsal hand veins (100 nmol/min (Vallance, et al., 1989); Chapter 4, Study 3) and forearm resistance vessels (4 μmol/min (Vallance, et al., 1989); Chapter 5, Study 2), as described above. The dose used in dorsal hand vein studies (100 nmol/min) has no effect on basal hand vein size (Vallance, et al., 1989). In contrast, in forearm

resistance vessels blockade of nitric oxide synthesis results in significant local vasoconstriction (Vallance, et al., 1989).

2.2.7 Aspirin

Aspirin (600 mg, soluble; Reckitt & Coleman, Hull, UK) was dissolved in 200 ml water and administered 30 min before local peptide infusions (Chapter 4, Study 3 and Chapter 5, Study 2). Aspirin irreversibly inhibits cyclo-oxygenase (EC 1.14.99.1) and, when given at this dose, inhibits bradykinin-stimulated endothelial production of prostacyclin by at least 85% with recovery developing over the next 6 hours (Heavey, et al., 1985).

2.2.8 L-753,037

L753,037 (Merck Sharpe and Dohme, Terlings Park, Middlesex, UK) is a potent nonpeptide, nonselective endothelin receptor antagonist (Zhao, et al., 1999) available in intravenous formulation. L-753,037 has almost balanced ET_A/ET_B selectivity; L-753,037 has threefold ET_A selectivity in cloned human endothelin receptors (K_iET_A 0.12 ± 0.01 nM, K_iET_B 0.17 ± 0.02 nM).

In Chapter 8, on separate occasions, L-753,037 (0.25 or 0.375 mg/kg) or matching placebo was administered by IV infusion for 30 min. L-753,037 was prepared under standard aseptic conditions within the hospital pharmacy department on the evening before administration and stored overnight at 4°C.

2.2.9 BMS-193884

BMS-193884 (Bristol-Myers Squibb Pharmaceutical Research Institute, Moreton, Wirral, UK) is a selective ET_A receptor antagonist available in orally active and intravenous formulation. BMS-193884 inhibits $^{[125I]}$ET-1 binding to recombinant human ET_A receptors in the nanomolar and ET_B receptors in the micromolar range

(K_iET_A 1.4 ± 0.1 nM, K_iET_B 18.8 ± 2 μM) (Unpublished data: Murugesan N *et al.* Biphenylsulfonamide endothelin receptor antagonists. 2. Discovery of 4'-Oxazolyl biphenylsulfonamides as a new class of potent, highly selective ET_A antagonists. Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 085343-5400, submitted for publication at time of submission of thesis).

Locally active administration - Intra-arterial infusion

BMS-193884 was prepared as a crystalline free acid and supplied as a 5 mg/ml aqueous solution for infusion. In Study 1, Chapter 9, placebo (0.9% saline, Baxter Healthcare Ltd, Therford, UK) or BMS-193884 (5 and 50 nmol/min) was administered double blind by continuous intra-arterial infusion for 60 min, according to the study randomisation schedule.

Although it is not possible to quantify exact tissue concentrations during local intra-arterial infusion, both doses of BMS-193884 were estimated to be within a range selective for the ET_A receptor (5 nmol/min ≈100 nM, 50 nmol/min ≈1 μM, assuming arterial blood flow ≈50 ml/min).

Systemically active administration - Oral administration

BMS-193884 was prepared as a crystalline free acid and supplied as a capsule dosage form for oral administration. BMS-193884 (50, 100 or 200 mg) or matching placebo capsules (Bristol-Myers Squibb Pharmaceutical Research Institute) were administered orally in Studies 2 and 3, Chapter 9, according to the study randomisation schedule, administration was double blind.

2.3 Haemodynamic measurements

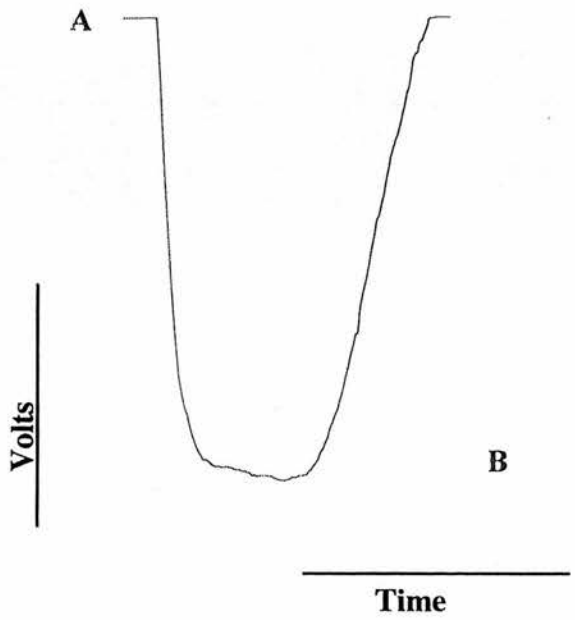
2.3.1 Dorsal hand vein diameter

Subjects rested semi-recumbent throughout. The left hand was supported above the level of the heart by means of an arm rest. Internal diameter of the dorsal hand vein, distended by inflation of an upper arm cuff to 30 mmHg, was measured by the technique of Aellig (Aellig, 1981) (Figure 2.4).

In brief, a magnetised lightweight rod rested on the summit of the infused vein approximately 1 cm downstream from the tip of the infusion cannula. This rod passed through the core of a linear variable differential transformer (LVDT; Model 025 MHR, Lucas Schaevitz Inc, Pennsauken, NJ, USA) supported above the hand by a small tripod, the legs of which rested on areas of the dorsum of the hand free of veins. If venoconstriction occurred while this cuff was inflated, or if the cuff was deflated with consequent emptying of the vein, there was a downward displacement of the lightweight rod which caused a linear change in the voltage generated by the LVDT. The voltage output from the LVDT was transferred to a Macintosh personal computer using a MacLab analogue-digital converter and Chart software (Figure 2.5).

Figure 2.4 The experimental set up for dorsal hand vein studies. The volunteer sits semi-recumbent in the bed, the hand is supported above the level of the heart.

Figure 2.5 Typical tracing obtained during a vein diameter recording. A denotes the voltage recorded during cuff inflation, B denotes the voltage recorded when the cuff is deflated, indicating subsequent emptying of the vein.



2.3.2 Forearm blood flow

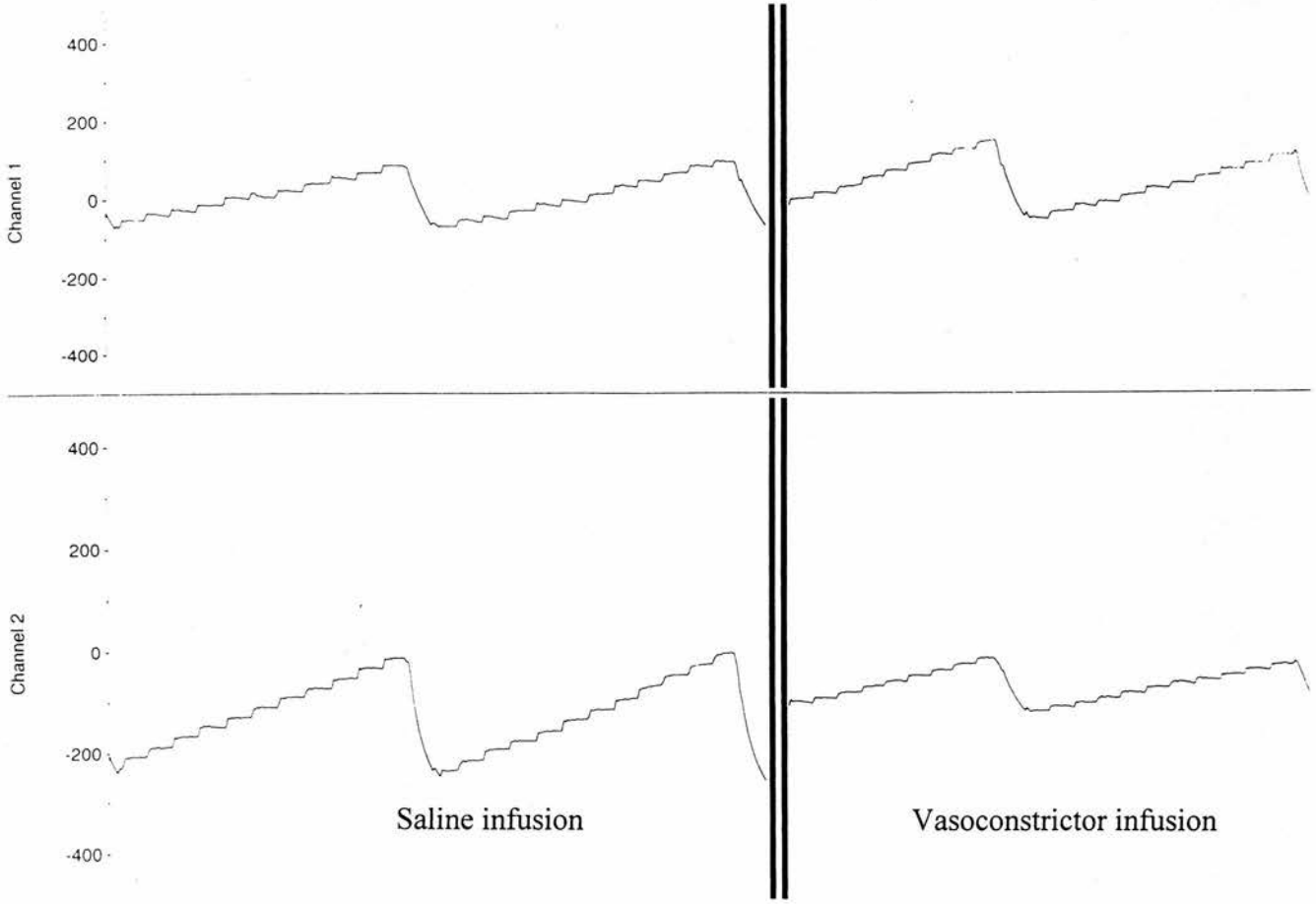
Subjects rested recumbent throughout each study. Blood flow was measured in the infused and non-infused forearms by venous occlusion plethysmography (Webb, 1995) using mercury-in-silastic strain gauges which were securely applied to the widest part of each forearm (Figure 2.6).

The hands were excluded from the circulation during each measurement period by inflation of a wrist cuff to 220 mmHg. Upper arm cuffs were intermittently inflated to 40 mmHg for 10 sec in every 15 sec to temporarily prevent venous outflow from the forearm and thus obtain plethysmographic recordings. Recordings of forearm blood flow were made repeatedly over 3 min periods unless otherwise stated. Venous occlusion plethysmography was performed using a dual channel strain gauge plethysmograph (Hokanson, USA) and calibration was achieved using the internal standard of the Hokanson plethysmography unit. The voltage output was transferred from the plethysmograph to a Macintosh personal computer (Classic II, Apple Computer Inc, Cupertino, CA) using a MacLab analogue-to-digital converter and Chart software (v. 3.2.8; both from AD Instruments, Castle Hill, NSW, Australia) (Figure 2.7).

The repeatability of the forearm blood flow response to intra-arterial infusion of ET-1 is described in full in Chapter 7.

Figure 2.6 The experimental set up for forearm blood flow studies. The volunteer lies recumbent in the bed, forearm blood flow is measured in both arms.

Figure 2.7 Traces obtained during forearm blood flow measurements, non-infused arm data are shown on Channel 1 and infused arm data on Channel 2. The left-hand section was recorded during baseline saline infusion, the right-hand section was recorded during infusion of a vasoconstrictor agent, vasoconstriction is indicated by the reduction in the gradient of the slope of the traces on Channel 2 (the infused arm).



2.3.3 Blood pressure and heart rate

Blood pressure and heart rate were measured in the non-infused arm using a well-validated semi-automated non-invasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan) (Wiinberg, et al., 1988).

Blood pressure was measured immediately after forearm blood flow or hand vein recordings to avoid any effect of the venous congestion caused by this procedure on these measurements (Patterson and Shepherd, 1954).

2.3.4 Cardiac function

Cardiac output and stroke volume were recorded by a well validated non-invasive bioimpedance technique (NCCOM3; BoMed Medical Manufacturer Ltd, Irvine, California, USA) (Thomas, 1992). The Bomed NCCOM3 (Bomed) is a non-invasive method of measuring cardiac function parameters by transthoracic bioimpedance. A constant sinusoidal current is applied through electrodes situated on the neck and trunk, to enable detection of changes in bioimpedance related to the cardiac cycle and blood flow. The Bomed has the facility to record cardiac output, stroke volume, ejection fraction, heart rate, peak blood flow, end-diastolic volume and ejection ratio. Cardiac output (CO, l/min) and stroke volume (SV, ml) are estimated from the measures of bioimpedance by the Sramek-Bernstein formula, adapted from the original formula of Kubicek (Thomas, 1992). These recordings can be corrected for body surface area and described as cardiac index (CI, l/min/m^2) and stroke index (SI, ml/m^2) (Haynes, et al., 1996). Total peripheral vascular resistance (TPVR) or total peripheral vascular index (TPVRI) can then be calculated as MAP divided by CO or CI, respectively, and expressed in arbitrary units (AU). Responses can be measured continuously or at specified timepoints following drug administration.

The Bomed provides a valuable tool for the assessment of within-subject changes in cardiac function in healthy volunteers, especially in acute interventional studies with drugs (de Mey and Belz, 1989). A number of studies have compared measurement of cardiac function by the Bomed technique with other non-invasive and invasive techniques, there is generally greater agreement between methods when the Bomed results are expressed as percentage change from baseline (Thomas, 1992).

2.4 Plasma assays

2.4.1 Plasma ET-1 and Big ET-1

In Chapters 8, 9 & 10, blood samples were obtained via an 18 SWG cannula sited in the non-infused arm. In brief, 10 ml samples were collected into sterile EDTA tubes (K3 EDTA, Vacutainer, Becton Dickinson Vacutainer Systems, Europe) centrifuged immediately at 2000 *g* for 20 min and stored in plain tubes at -80°C prior to assay. Endothelin-1 and big ET-1 (Peninsula Laboratories Europe) were determined by standard radioimmunoassay, as described previously (Hand, et al., 1999; Newby, et al., 1998).

2.4.2 Plasma BMS-193884

In Chapter 9, Study 2 and Study 3, blood samples for assay of BMS-193884 and its metabolites were obtained, via an 18 SWG cannula sited in the non-infused arm. In brief, 10 ml samples were collected into sterile EDTA tubes (K3 EDTA, Vacutainer, Becton Dickinson Vacutainer Systems, Europe) centrifuged immediately at 1000 *g* for 15 min (BMS-193884 assay) and stored in plain tubes at -80°C prior to assay.

Concentrations of BMS-193884 and its metabolites, BMS-205868 and BMS-212442, were determined by a well validated standard liquid chromatography/mass spectrometry method (unpublished data[2]: Eades DM. To develop an LC/MS/MS

method for the quantitative determination of BMS-193884 and two of its metabolites in various matrices. AAPS Proceedings, 1997. AAPS Meeting, November 1, 1997).

2.5 Statistical analysis

2.5.1 Dorsal hand vein diameter

Baseline vein diameter was calculated as the mean of the last 3 measurements during the saline infusion, before the start of the active drug infusion, and is expressed in arbitrary units. In order to minimise the effects of any inter-subject and inter-study variability in hand vein diameter, responses following infusion of peptides are expressed as percentage change in vein diameter from baseline (Haynes, et al., 1994).

2.5.2 Forearm blood flow

Plethysmographic data listings were extracted from data files and forearm blood flows calculated for individual venous occlusion cuff inflations using a template spreadsheet (Excel 5.0; Microsoft Ltd, Wokingham, UK). Recordings made in the first 60 sec after wrist cuff inflation were not used for analysis because of the transient instability in blood flow that this causes (Kerslake, 1949). Blood flow in both forearms was obtained from the mean of the last 5 consecutive recordings of each measurement period. Baseline blood flow was taken as the last measurement during the saline infusion, before the start of the active drug infusion. Forearm blood flow results are expressed as the % change from baseline in the ratio of blood flow between the infused and non-infused arms (Webb, 1995).

An investigation of the repeatability of the assessment of forearm blood flow response to intra-arterial infusion of ET-1 and comparison of methods of data presentation are discussed in more detail in Chapter 7.

2.5.3 General

Statistical analysis methods for blood pressure and heart rate, cardiac function and plasma concentrations of ET-1 and BMS-193884 are described in future chapters.

All results are expressed as mean ± standard error of the mean (SEM). Blood pressure, heart rate and baseline measurements during local infusion studies were compared using the Student's paired *t*-test. The forearm blood flow and vein diameter responses were examined by repeated-measures analysis of variance (ANOVA) using StatView 512+ software (Brainpower Inc, Calabasas, CA, USA or Excel 5.0 (Microsoft Ltd, Wokingham, UK). Statistical significance was accepted at the 5% level.

Footnote

Unpublished data[1]: Murugesan N *et al.* Biphenylsulfonamide endothelin receptor antagonists. 2. Discovery of 4'-Oxazolyl biphenylsulfonamides as a new class of potent, highly selective ET_A antagonists. Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 085343-5400, submitted for publication at time of submission of current manuscript.

Unpublished data[2]: Eades DM. To develop an LC/MS/MS method for the quantitative determination of BMS-193884 and two of its metabolites in various matrices. AAPS Proceedings, 1997. AAPS Meeting, November 1, 1997.

Chapter 3

The local constrictor effects of endothelin receptor agonists in capacitance vessels *in vivo*.

Endothelin ETA and ETB receptors cause vasoconstriction of human resistance and capacitance vessels in vivo. Haynes WG, Strachan FE, Webb DJ. Circulation 1995;92:357-63.

Constriction to ETB receptor agonists, BQ-3020 and sarafotoxin S6c, in human resistance and capacitance vessels *in vivo*. Strachan FE, Crockett TR, Mills NM, Gray GA, Webb DJ. Br J Clin Pharmacol 2000;50:27-30

3.1 Introduction

As discussed in Chapter 1, the powerful vasoconstrictor and vasopressor effects of endothelin-1 (ET-1) (Clarke, et al., 1989; Yanagisawa, et al., 1988) are predominantly mediated via the ET-1 selective, vascular smooth muscle cell ET_A receptor (Arai, et al., 1990). ET_B receptors have also been described on vascular smooth muscle cells (Davenport, et al., 1993) and may contribute to the vasoconstrictor effects of ET-1 (Clozel, et al., 1992). Indeed, in animals and in humans there is functional evidence for ET_B mediated vasoconstriction *in vitro*, particularly in veins (Moreland, et al., 1994; Seo, et al., 1994; Sumner, et al., 1992).

The contribution of ET_B receptors to vasoconstriction is variable and appears to depend markedly on species, vessel type and vessel size (Davenport and Maguire, 1994). Furthermore, the functional significance of the vascular smooth muscle ET_B receptor in humans is unclear; with *in vitro* studies reporting that ET_B receptors make either a minimal (Davenport and Maguire, 1994; Fukuroda, et al., 1994; Godfraind, 1993; Maguire, et al., 1994; Riezebos, et al., 1994) or, at most, a moderate contribution (Seo, et al., 1994; White, et al., 1994) to vasoconstriction, depending on the types of vessel studied. Given that species differences in ET_B responses exist (Reynolds, et al., 1995), it is important to investigate the function of endothelin ET_A and ET_B receptors in blood vessels *in vivo* in humans. As a first step in this investigation, a series of studies assessing the responses to infusion of locally active doses of endothelin receptor agonists in human hand veins *in vivo* were performed. Investigation of locally active doses allows early and safe investigation of the effects of these peptides in humans *in vivo* with the distinct advantage of studying these effects in intact vessels, under physiological conditions, without the confounding haemodynamic effects of systemically active doses.

The vascular effects of the ET_A and ET_B receptor were investigated using ET-1 as a non-selective ET receptor agonist and SFTX6c and BQ-3020 as ET_B receptor selective agonists (for structures see Figure 1.2 and 2.3).

3.2 Methods

3.2.1 Subjects

Six healthy male subjects within the age range of 18-60 years were recruited to Study 1, six were recruited to Study 2 and eight were recruited to Study 3, under the standard conditions listed in Chapter 2.

3.2.2 Drugs

Study 1

Endothelin-1 (5 pmol/min) and SFTX6c (5 pmol/min) were administered by continuous infusion for 60 min.

Study 2

Sarafotoxin S6c was administered by continuous infusion for 90 min at an infusion rate of 5 or 50 pmol/min.

Study 3

Sarafotoxin S6c (5 pmol/min) and BQ-3020 (50 pmol/min) were administered by continuous infusion for 90 min.

In each of the studies, saline was infused for 30 min before infusion of the study agent, the total infusion rate was kept constant at 0.25ml/min.

3.2.3 Measurements

Dorsal hand vein diameter was measured in each study at 5 min intervals throughout, as described in Chapter 2.3.1.

Blood pressure was measured at 30 min intervals in each study, as described in Chapter 2.3.3.

3.2.4 Study design

Studies were performed single blind, with the experimental subjects, but not the investigators, blinded to the peptide and dose administered in each study.

Study 1 Intra-venous endothelin-1 and sarafotoxin S6c

The local effects of intra-venous ET-1 and SFTX6c were investigated in six healthy men in a single-blind, randomised, 2 way crossover study. Subjects were studied on 2 separate occasions, in random, balanced order. Endothelin-1 or SFTX6c was infused at 5 pmol/min for 60 min.

Study 2 Intra-venous sarafotoxin S6c

The local effects of intra-venous SFTX6c were investigated in six healthy men in a single-blind, randomised, 2 way crossover study. Subjects were studied on 2 separate occasions, in random, balanced order. Sarafotoxin S6c was infused at 5 or 50 pmol/min for 90 min.

Study 3 Intra-venous sarafotoxin S6c and BQ-3020

The local effects of intra-venous and intra-arterial (see Chapter 5, Study 1) infusion of BQ-3020 and SFTX6c were investigated in eight healthy male subjects in a single-blind, randomised, 4 way crossover study. Subjects attended for 4 visits, each separated by at least one week. On separate occasions, in a random order, each subject

received either intra-venous infusion of SFTX6c (5 pmol/min) or BQ-3020 (50 pmol/min) for 90 min, or intra-arterial infusion of SFTX6c (5 pmol/min) or BQ-3020 (50 pmol/min), the intra-arterial studies are discussed in Chapter 5.

3.2.5 Data analysis and statistics

Baseline vein diameter was calculated as the mean of the last 3 measurements during the saline infusion, before the start of the active drug infusion. Hand vein diameter results are expressed as the percentage change in vein diameter from baseline (see Chapter 2.5.1).

All results are expressed as mean ± standard error of the mean (SEM) at the end of the infusion: 60 min for Study 1 and 90 min for Study 2 & 3. Vein diameter responses were examined by repeated-measures analysis of variance (ANOVA). Statistical significance was accepted at the 5% level.

3.3 Results

Six healthy male subjects (age range 22 - 39 years) completed Study 1, six (age range 19-22 years) completed Study 2 and eight (age range 20 - 28 years) completed Study 3. There was no significant difference between baseline measurements on each of the study visits. There was no significant change in blood pressure and heart rate in the non-infused arm at the end of each infusion, confirming that any drug effects were confined to the infused arm (Table 3.1).

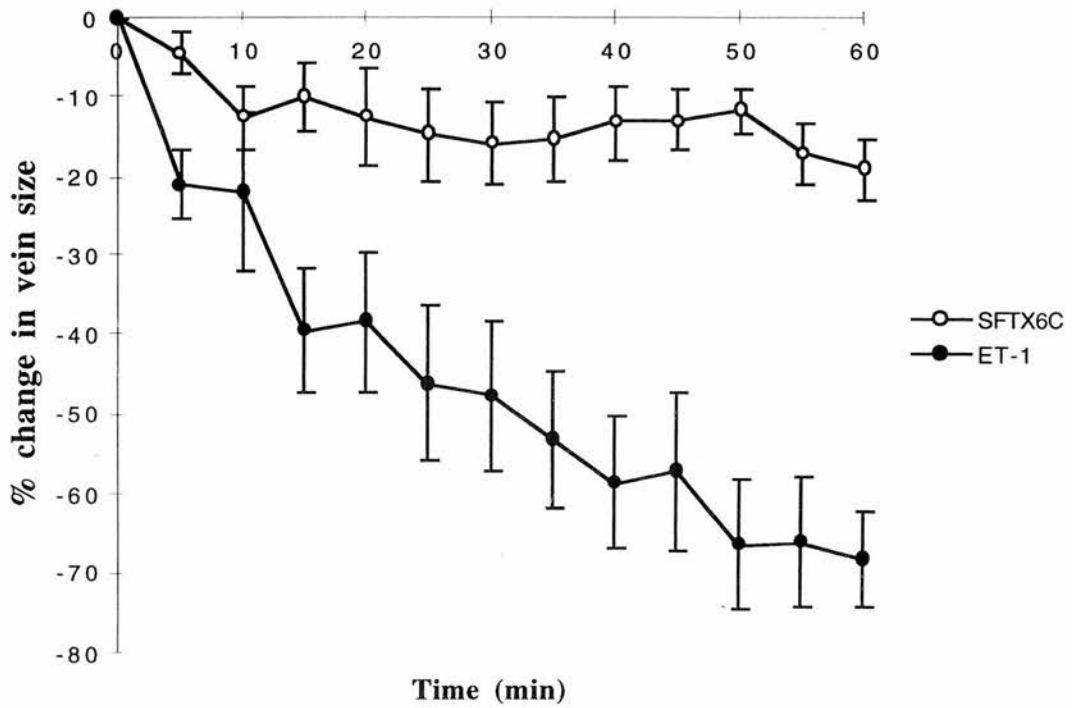
Table 3.1 Mean arterial pressure (MAP), heart rate (HR), forearm blood flow (FBF) and vein diameter at baseline and at the end of each infusion (Study 1, 60 min; Study 2, 90 min; Study 3; 90 min). Values are mean ± SEM.

	Study 1		**Study 2**		**Study 3**	
	ET-1	**SFTX6c**	**SFTX6c**	**SFTX6c**	**SFTX6c**	**BQ-3020**
	(5 pmol/min)	(5 pmol/min)	(5 pmol/min)	(50 pmol/min)	(5pmol)	(50pmol)
MAP (mmHg)						
Basal	89±5	84±3	85±2	88±2	87±2	88±2
end of infusion	89±4	84±2	90±2	87±2	87±2	90±4
HR (bpm)						
Basal	70±4	65±4	65±4	68±6	58±3	57±3
end of infusion	68±5	59±5	59±4	64±5	56±4	59±4
Vein Diameter (arbitrary units)						
Basal	0.9±0.2	1.1±0.7	5.1±1.1	5.3±0.8	2.7±0.4	2.6±0.3
end of infusion	0.3±0.1	0.9±0.2	4.6±1.1	5.0±0.8	2.3±0.3	1.8±0.3

Study 1 Intra-venous endothelin-1 and sarafotoxin S6c

Endothelin-1 caused a marked decrease in hand vein diameter (-68±6%, p=0.001) [Figure 3.1] which was slow in onset. Sarafotoxin S6c also caused venoconstriction, although the decrease in hand vein diameter (-19±4%, p=0.003) [Figure 3.1] was significantly less than that to ET-1 (p=0.002).

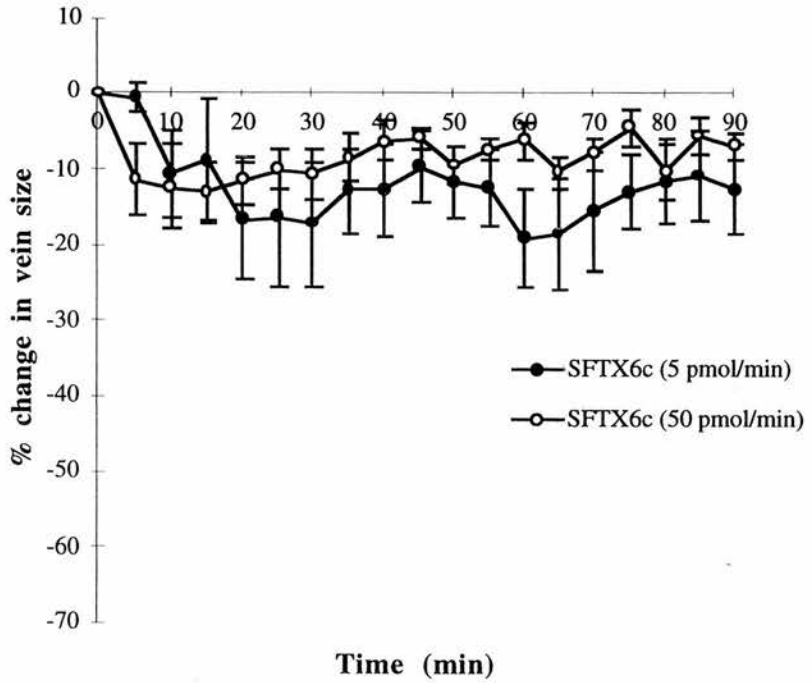
Figure 3.1 Response of hand vein diameter to local intravenous infusion of ET-1 (5 pmol/min; closed circles) and SFTX6c (5 pmol/min; open circles). Responses are expressed as mean % change in blood flow ratio ± SEM.



Study 2 Intra-venous sarafotoxin S6c, at 2 dose levels

Sarafotoxin S6c caused venoconstriction at both doses (-13±6%, p=0.03; 5 pmol/min and -7±2%, p<0.01; 50 pmol/min) [Figure 3.2]. There was no significant difference between the responses to 5 and 50 pmol/min (p=0.5), although the response to SFTX6c (5 pmol/min) appeared to be greater than SFTX6c (50 pmol/min) after the first 15 min of the infusion.

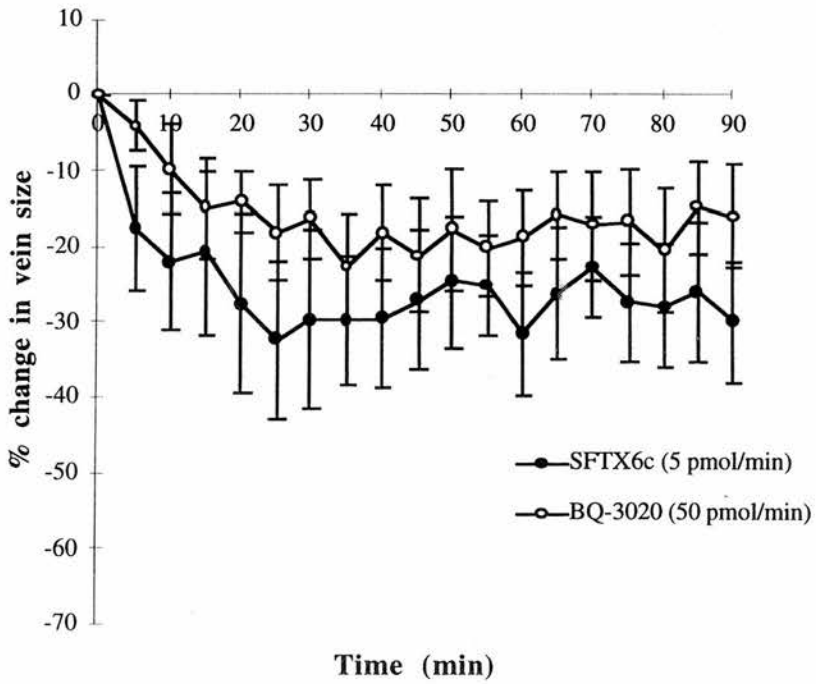
Figure 3.2 Response of hand vein diameter to local intravenous infusion of SFTX6c (5 pmol/min, closed circles; and 50 pmol/min, open circles). Responses are expressed as mean % change in blood flow ratio ± SEM.



Study 3 Intra-venous sarafotoxin S6c and BQ-3020

Sarafotoxin S6c (5pmol/min) caused a significant reduction in vein diameter (-30±8%, $p<0.001$). BQ-3020 (50 pmol/min) also caused a small, but significant, venoconstriction (-16±7%; $p<0.001$) [Figure 3.3]. For both agonists, the response reached a maximum after ~30 min and was sustained for the rest of the infusion. There was a small but significant difference between the hand vein responses to SFTX6c and BQ-3020 ($p=0.001$).

Figure 3.3 Response of hand vein diameter to local intravenous infusion of SFTX6c (5 pmol/min; closed circles) and BQ-3020 (50 pmol/min; open circles), respectively. Responses are expressed as mean % change in blood flow ratio ± SEM.



3.4 Discussion

These results show that the non-selective ET receptor agonist ET-1 constricts hand capacitance vessels *in vivo*, consistent with earlier reports of vasoconstrictor effects of ET-1 in forearm resistance vessels *in vivo* (Clarke, et al., 1989). In addition, the demonstration of local constrictor effects of the ET_B receptor selective agonists SFTX6c and BQ-3020, in capacitance vessels, support the hypothesis that ET_B receptors can, under some circumstances, mediate vasoconstriction and may contribute to the vasoconstrictor effects of endothelin-1 (Clozel, et al., 1992).

Interestingly, there was no significant difference between the responses to the two dose levels of SFTX6c. Indeed, in Study 2, the constrictor response to SFTX6c tended to be less with the higher dose level (50 pmol/min) than the response to the lower dose level (5 pmol/min). This may be due to tachyphylaxis of the ET_B receptor following exposure to the higher concentration of SFTX6c. Tachyphylaxis of the ET_B receptor has been described in *in vitro* studies (Sudjarwo, et al., 1994) at concentrations of ~100 nM. Based on venous blood flow of 1-5 ml/min, the expected tissue concentrations during the 50 pmol/min infusion, would be 10 - 50 nM, therefore, there may be some degree of tachyphylaxis at this dose level. It is also possible that the higher dose infusion in the current study mediates release of endothelium-derived dilators nitric oxide (Tsukahara, et al., 1994) and prostacyclin (DeNucci, et al., 1988) which could offset the constrictor effects of SFTX6c.

There has been some uncertainty over the use of SFTX6c as an agonist in the investigation of the endogenous effects of the ET_B receptor *in vivo* (Flynn, et al., 1995; Shraga-Levine, et al., 1994;Sokolovsky, 1995). The exceptionally high degree of selectivity of SFTX6c for the ET_B receptor may result in effects not likely to be seen with binding of the non-selective endogenous ligand, ET-1. Additional concerns have arisen as a result of the finding that while SFTX6c, binds to the same receptors as the

endogenous ligands, ET-1 and ET-3, its actions are achieved via a different signalling pathway (Shraga-Levine, et al., 1994). In Study 3, constrictor effects were observed with SFTX6c and a structurally distinct ET_B receptor selective agonist, BQ-3020. The fact that constriction also occurs in response to BQ-3020 provides evidence that the effects of SFTX6c are not idiosyncratic to this peptide and may be representative of endogenous binding of ET-1 to the ET_B receptor. Indeed, constrictor effects of the endogenous ET_B selective agonist ET-3, have also been described in the hand vein (Ferro, et al., 2000). Therefore, concerns over signalling pathway differences between SFTX6c and endothelin peptide binding (Shraga-Levine, et al., 1994;Sokolovsky, 1995) may not be functionally important.

In study 3, the response to BQ-3020 was less than that to SFTX6c. Although, the reason for this difference is unclear, it is important to note that the doses of agonists selected for the study were not intended to be directly comparable in terms of activity at the receptor site. It is unlikely that this difference is functionally significant.

An informal observation noted from Study 2 and 3 is that there is a higher degree of variability in the response to ET_B receptor agonists between subjects than with the response to ET-1, with some subjects demonstrating little or no response and others demonstrating >40% constriction. This variability has also been discussed following review of the distribution and function of ET_A and ET_B receptors (Davenport and Maguire, 1994). It could be that there is more variation in the distribution of vascular smooth muscle cell ET_B receptors within the population and therefore, that ET_B mediated constriction is more important in some individuals or patient groups than others. Indeed, upregulation of the ET_B receptor has been suggested in some patient groups (Kanno, et al., 1993).

The constrictor effects described with ET_B receptor agonists are likely to be caused by stimulation of vascular smooth muscle ET_B receptors. However, there are a number of possible alternative explanations, ET_B receptors may cause late onset vasoconstriction through stimulation of the generation of endothelium-derived vasoconstrictor agents. These substances might include constrictor prostanoids or even endothelin-1, because endothelin-3 is known to stimulate production of endothelin-1 *in vitro* (Yokokawa, et al., 1991). It is also possible that ET_B agonists might prevent local clearance of ET-1, resulting in increased circulating ET-1 which could then act on unopposed ET_A receptors to cause vasoconstriction. However, this possibility is unlikely, given that ET_A antagonists do not appear to influence vasoconstriction to SFTX6c *in vitro* (Gray, et al., 1994; Moreland, et al., 1992; Seo, et al., 1994; Sudjarwo, et al., 1994; Warner, et al., 1993) and that a proportion of the response to ET-1 appears to be resistant to ET_A receptor selective antagonists (Seo, et al., 1994; Sudjarwo, et al., 1994).

It could also be postulated that some of the effects of ET_B receptor agonists are mediated by a putative ET_C (ET-3 selective) receptor. However, although there is evidence from binding (Yokokawa, et al., 1991) and functional studies (Harrison, et al., 1992) to support the existence of an endothelin-3 selective receptor in the vasculature, and a potential candidate has been identified in *Xenopus laevis* melanophores (Karne, et al., 1993), this receptor has not been identified in humans. Any contribution from the putative ET_C receptor will depend on its isolation and pharmacological characterization.

The results of the current studies confirm the vasoconstrictor effects of ET-1 *in vivo* and indicate a potential role for the ET_B receptor in the mediation of this constriction. Although vasoconstriction to the ET_B agonists has been described, the effects of ET_B receptor agonists may not give a true reflection of the overall balance of the endogenous effects of ET-1 mediated by ET_B receptors. Studies with selective ET_B

receptor antagonists should help to clarify this issue further, although, such agents may potentiate responses to ET-1, through reduced clearance of ET-1 and subsequent increases in circulating ET-1.

In future chapters, I will discuss the results of studies investigating the haemodynamic effects of endothelin antagonists *in vivo* and discuss how these results contribute to our understanding of the relative contributions of each receptor subtype to ET-1 mediated vascular tone.

Chapter 4

The role of the endothelium-derived dilators and endothelin receptors in the local constrictor effects of endothelin receptor agonists in capacitance vessels *in vivo*

Endothelium-dependent modulation of venoconstriction to sarafotoxin S6c in human hand veins *in vivo*. Strachan FE, Haynes WG, Webb DJ. J Cardiovasc Pharmacol 1995;26:S180-S182.

4.1 Introduction

The constrictor effects of ET_B receptor selective agonists in hand veins *in vivo* are described in Chapter 3 (Haynes, et al., 1995;Strachan, et al., 2000). However, the functional significance of the vascular effects of ET_B receptor selective agonists and the contribution of ET_B receptors to ET-1 mediated vasoconstriction remains unclear. Vasoconstriction to ET-1 is attenuated but not completely abolished following administration of the ET_A receptor antagonists BQ-123 (McMurdo, et al., 1993) and FR139317 (Gardiner, et al., 1994; Seo, et al., 1994). However, the attenuation of the ET-1 response with FR139317 was greater following desensitisation of the ET_B receptors with preincubation with SFTX6c (Seo, et al., 1994). These results indicate a potential contribution from the ET_B receptor to ET-1 mediated constriction.

In addition to the proposed constrictor effects of the vascular smooth muscle ET_B receptor, the endothelial ET_B receptor stimulates generation of nitric oxide and dilator prostaglandins (DeNucci, et al., 1988;Tsukahara, et al., 1994) to mediate vasodilatation to modulate the contrictor effects of ET-1. Indeed the constrictor effects of the ET_B receptor selective agonists IRL 1620 and ET-3 were greater in endothelium denuded vessels than in endothelium intact (Shetty, et al., 1993). In addition, in human hand veins, venoconstriction to ET-1 is attenuated by dilator prostanoids but not by nitric oxide (Haynes and Webb, 1993).

In order to identify the relative contribution of each receptor subtype to ET-1 mediated vascular tone, the responses to the non-selective agonist ET-1, and the ET_B receptor selective agonist SFTX6c were assessed in the current series of studies (Study 1, 2 and 3), in the presence and absence of locally active doses of the ET_A receptor selective antagonist BQ-123 and the ET_B receptor selective antagonist BQ-788, in the dorsal hand vein *in vivo*.

In addition, the effect of the endothelium-derived dilators, nitric oxide and prostacyclin, on ET_B receptor mediated constriction was investigated in a further study (Study 4), using the ET_B receptor agonist SFTX6c, the cyclo-oxygenase inhibitor, aspirin, and the nitric oxide synthase inhibitor, L-NMMA.

4.2 Methods

4.2.1 Subjects

Six healthy male subjects within the age range of 18-60 years were recruited to each study, under the standard conditions listed in chapter 2.

4.2.2 Drugs

Study 1

Endothelin-1 (5 pmol/min), SFTX6c (5 pmol/min) and BQ-123 (0.3 nmol/min) were administered by continuous infusion for 60 min, as described in Chapter 2.

Study 2

Endothelin-1 (5 pmol/min), BQ-123 (1 nmol/min) and BQ-788 (1 nmol/min) were administered by continuous infusion for 90 min, as described in Chapter 2.

Study 3

Sarafotoxin S6c (5 pmol/min) and BQ-788 (1 nmol/min) were administered by continuous infusion for 60 min, as described in Chapter 2.

Study 4

Sarafotoxin S6c (5 pmol/min) and L-NMMA (100 nmol/min) were administered by continuous infusion for 60 min. Aspirin (600 mg) was administered orally 30 min before the SFTX6c infusion, as described in Chapter 2.

In each of the studies, saline was infused for 30 min before infusion of the active infusion, the total infusion rate was kept constant at 0.25ml/min.

4.2.3 Measurements

Dorsal hand vein diameter was measured in each study at 5 min intervals throughout, as described in Chapter 2.3.1.

Blood pressure was measured at 30 min intervals in each study, as described in Chapter 2.3.3.

4.2.4 Study design

Studies were performed single blind, with the experimental subjects, but not the investigators, blinded to the peptide and dose administered in each study. Oral administration of Aspirin was unblinded.

Study 1 Intra-venous endothelin-1, sarafotoxin S6c and BQ-123

The local effects of intra-venous ET-1, SFTX6c and BQ-123 were investigated in six healthy men in a single-blind, randomised, 5 way crossover study. On separate occasions, ET-1 or SFTX6c was infused at 5 pmol/min for 60 min either alone or in combination with BQ-123 (0.3 nmol/min), in addition, BQ-123 was infused alone. Subjects were studied on 5 separate occasions, in random, balanced order, each study period was separated by at least one week.

Study 2 Intra-venous endothelin-1, BQ-123 and BQ-788

The local effects of intra-venous ET-1, BQ-123 and BQ-788 were investigated in six healthy men in a single-blind, randomised, 3 way crossover study. On separate occasions, ET-1 was infused at 5 pmol/min for 90 min either alone or in combination with BQ-123 (1 nmol/min) or BQ-788 (1 nmol/min). Subjects were studied on 3 separate occasions, in random, balanced order, each study period was separated by at least one week.

Study 3 Intra-venous sarafotoxin S6c and BQ-788

The local effects of intra-venous SFTX6c and BQ-788 were investigated in six healthy men in a single-blind, randomised, 3 way crossover study. On separate occasions, SFTX6c (5 pmol/min) or BQ-788 (1 nmol/min) were infused for 60 min either alone or in combination. Subjects were studied on 3 separate occasions, in random, balanced order, each study period was separated by at least one week.

Study 4 Intra-venous sarafotoxin S6c and L-NMMA with Aspirin

The local effects of intra-venous SFTX6c were investigated during co-infusion of L-NMMA or following oral administration of Aspirin, in a single-blind, 4 way crossover study. Subjects were studied on 4 separate occasions, each study period was separated by at least one week. The order of the study periods was randomised, with the exception of the final study period in which the effects of SFTX6c were investigated in the presence of both L-NMMA and Aspirin.

4.2.5 Data analysis and statistics

Baseline vein diameter was calculated as the mean of the last 3 measurements during the saline infusion, before the start of the active drug infusion. Hand vein diameter results are expressed as the percentage change in vein diameter from baseline (see Chapter 2).

All results are expressed as mean ± standard error of the mean (SEM) at the end of the infusion: 60 min for Study 1, 3 & 4 and 90 min for Study 2. Statistical significance was accepted at the 5% level.

4.3 Results

Six subjects (age range 26 - 39 years) completed Study 1, six (age range 19-22 years) completed Study 2, six (age range 20 - 28 years) completed Study 3 and six (age range 22-39 years) completed Study 4. There was no significant difference between baseline measurements on each of the study visits. There was no significant change in blood pressure and heart rate in the non-infused arm at the end of each infusion, confirming that any drug effects were confined to the infused arm (Tables 4.1 - 4.3).

Table 4.1 Mean arterial pressure (MAP), heart rate (HR), and vein diameter at baseline and at the end of each infusion (60 min) for Study 1. Values are mean ± SEM.

	Study 1				
	ET-1 (5 pmol/min)	**ET-1 + BQ-123** (0.3 nmol/min)	**SFTX6c** (5 pmol/min)	**SFTX6c + BQ-123** (0.3 nmol/min)	**BQ-123** (0.3 nmol/min)
MAP (mmHg)					
Basal	84±3	86±4	88±3	86±3	83±2
end of infusion	84±5	87±4	89±4	85±3	81±3
HR (bpm)					
Basal	68±4	64±2	67±4	68±4	67±4
end of infusion	67±4	63±3	65±4	66±2	65±3
Vein Diameter (arbitrary units)					
Basal	1.2±0.3	1.2±0.2	1.3±0.3	1.7±0.5	1.2±0.2
end of infusion	0.6±0.3	1.0±0.2	1.1±0.2	1.6±0.5	1.1±0.2

Table 4.2 Mean arterial pressure (MAP), heart rate (HR), and vein diameter at baseline and at the end of each infusion: 90 min for Study 2 and 60 min for Study 3. Values are mean ± SEM.

	Study 2			Study 3		
	ET-1 (5 pmol/min)	**ET-1 + BQ-123** (1 nmol/min)	**ET-1 + BQ-788** (1 nmol/min)	**SFTX6c** (5 pmol/min)	**SFTX6c + BQ-788** (1 nmol)	**BQ-788** (1 nmol)
MAP (mmHg)						
Basal	87±4	86±2	85±5	81±4	82±3	81±3
end of infusion	84±3	85±2	87±2	82±4	83±5	80±3
HR (bpm)						
Basal	64±3	63±4	66±3	59±5	59±4	60±4
end of infusion	61±3	65±4	67±4	55±5	58±4	59±4
Vein Diameter (arbitrary units)						
Basal	2.4±0.6	2.3±0.8	2.3±0.8	5.1±1.4	3.3±1.3	4.8±1.2
end of infusion	1.7±0.7	2.0±0.7	1.7±0.8	4.6±1.4	3.4±1.4	4.6±1.2

Table 4.3 Mean arterial pressure (MAP), heart rate (HR), and vein diameter at baseline and at the end of each infusion (60 min) for Study 4. Values are mean ± SEM.

	SFTX6c (5 pmol/min)	**SFTX6c + Aspirin** (600 mg)	**SFTX6c + L-NMMA** (100 nmol/min)	**SFTX6c + Aspirin + L-NMMA**
MAP (mmHg)				
Basal	84±3	87±3	87±3	87±3
end of infusion	84±2	89±4	89±2	88±3
HR (bpm)				
Basal	65±4	63±4	66±4	62±5
end of infusion	59±5	61±4	66±3	60±4
Vein Diameter (arbitrary units)				
Basal	1.1±0.7	0.9±0.1	1.2±0.3	0.9±0.2
end of infusion	0.9±0.2	0.6±0.1	0.9±0.3	0.5±0.1

Study 1 Intra-venous endothelin-1, sarafotoxin S6c and BQ-123

Endothelin-1 caused a slow-onset and marked decrease in hand vein diameter (-61±12%, p=0.0008) which was significantly attenuated in the presence of BQ-123 (p<0.001 vs ET-1) (Figure 4.1a), although there was still a reduction in vein diameter in the presence of BQ-123 (-20±7%, p<0.001 vs basal). Sarafotoxin S6c also reduced hand vein diameter (-14±5%, p<0.001 vs basal), similarily, there was a reduction in vein diameter during co-infusion of SFTX6c and BQ-123 (-19±10%, p<0.001) (Figure 4.1b) which did not differ significantly from the response to SFTX6c alone (p=0.2). Interestingly the response to ET-1 in the presence of BQ-123 was not significantly different from the response to SFTX6c alone (-20±7% vs -14±5%, respectively; p=0.4) (Figure 4.1c). There was a small reduction in vein size following infusion of BQ-123 alone (-7±3%), although small, this change was found to be significant (p=0.03 vs basal).

Figure 4.1a Response of hand vein diameter to local intravenous infusion of ET-1 (5 pmol/min) alone (closed circles) and during co-infusion with BQ-123 (0.3 nmol/min; open circles). Responses are expressed as mean % change in blood flow ratio ± SEM.

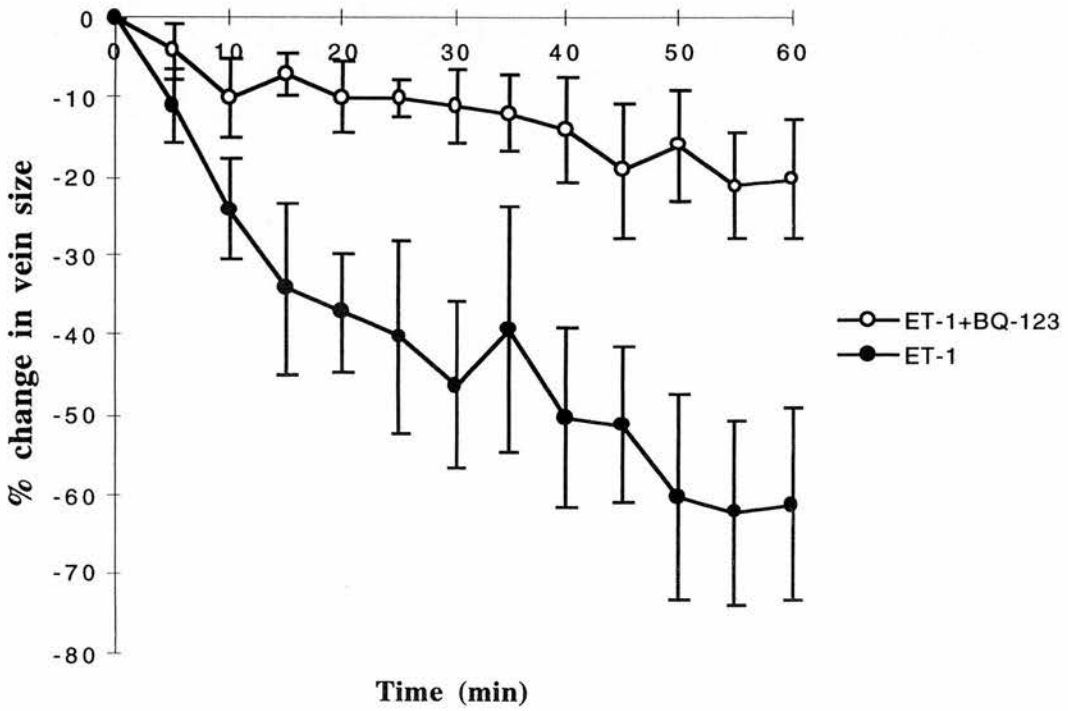


Figure 4.1b Response of hand vein diameter to local intravenous infusion of SFTX6c (5 pmol/min) alone (closed squares) and during co-infusion with BQ-123 (0.3 nmol/min; open squares). Responses are expressed as mean % change in blood flow ratio ± SEM.

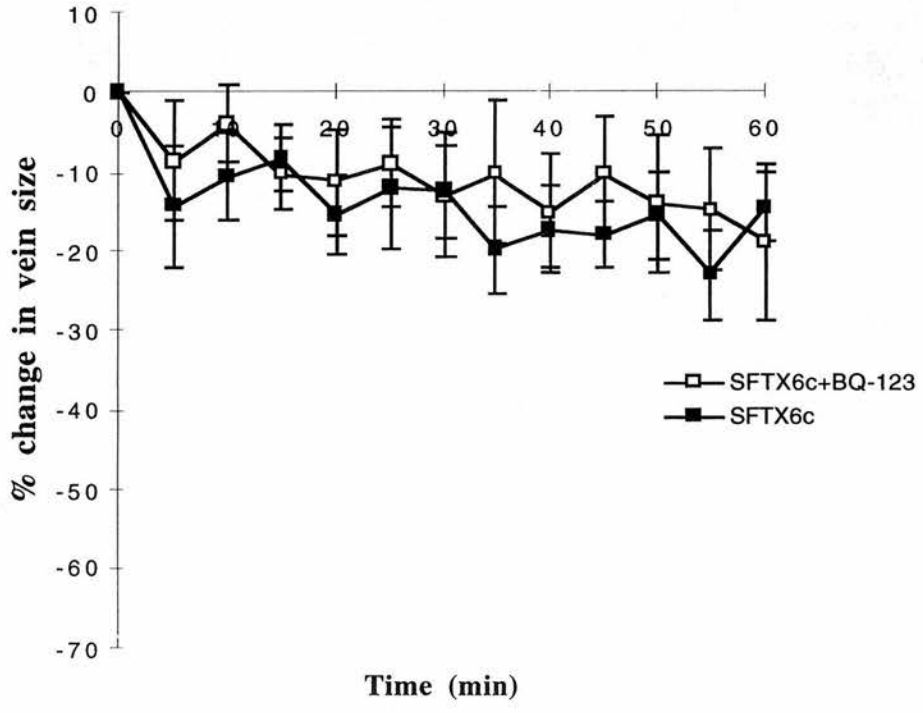
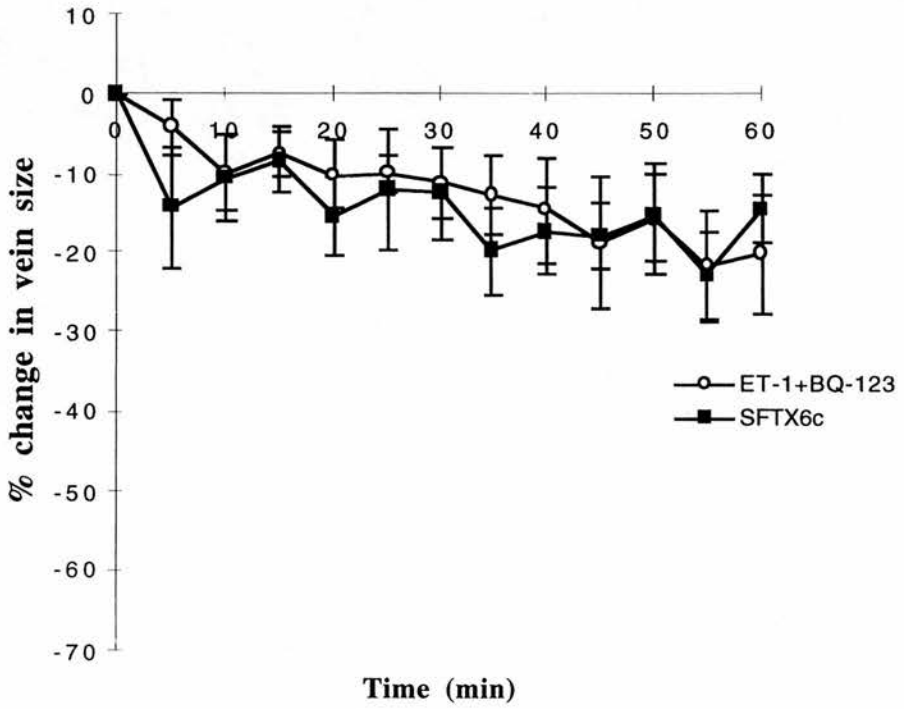


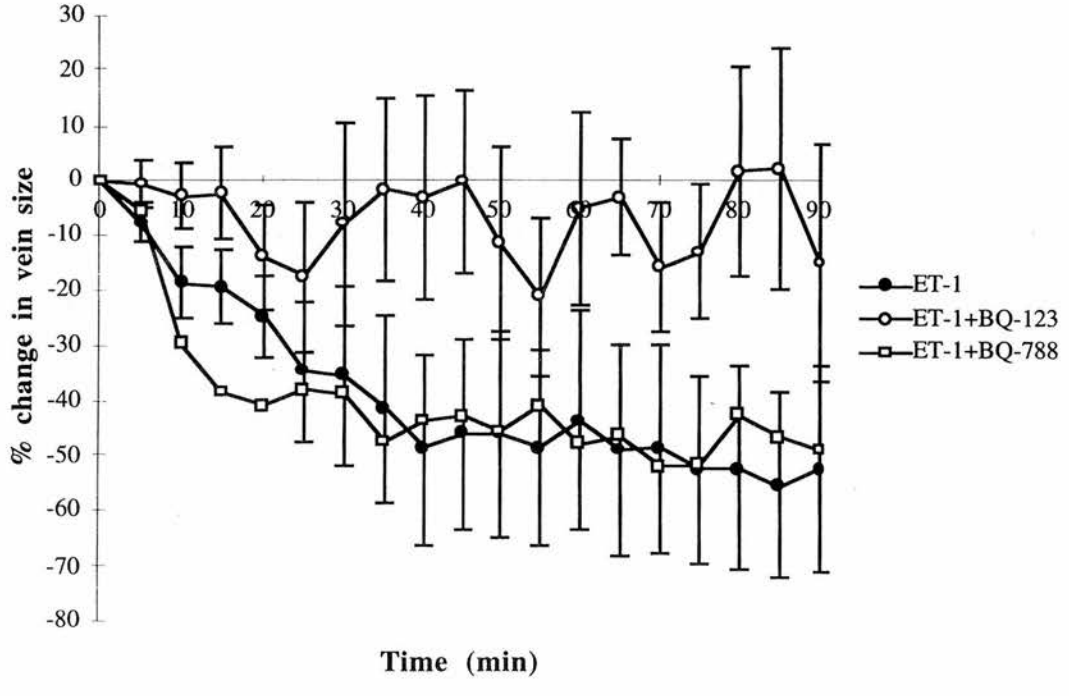
Figure 4.1c Response of hand vein diameter to local intravenous infusion of ET-1 (5 pmol/min) during co-infusion with BQ-123 (0.3 nmol/min; open circles) and SFTX6c (5 pmol/min) alone (closed squares). Responses are expressed as mean % change in blood flow ratio ± SEM.



Study 2 Intra-venous endothelin-1, BQ-123 and BQ-788

Endothelin-1 caused a slow-onset and marked decrease in hand vein diameter (-52±19%, $p<0.001$) which was attenuated in the presence of BQ-123 (-14±22%; $p<0.001$ vs ET-1, $p=0.04$ vs basal) (Figure 4.2). In contrast, BQ-788 had no effect on the response to ET-1, causing a significant reduction in hand vein diameter (-49±18%, $p<0.001$) which was not significantly different from the response to infusion of ET-1 alone ($p=0.8$).

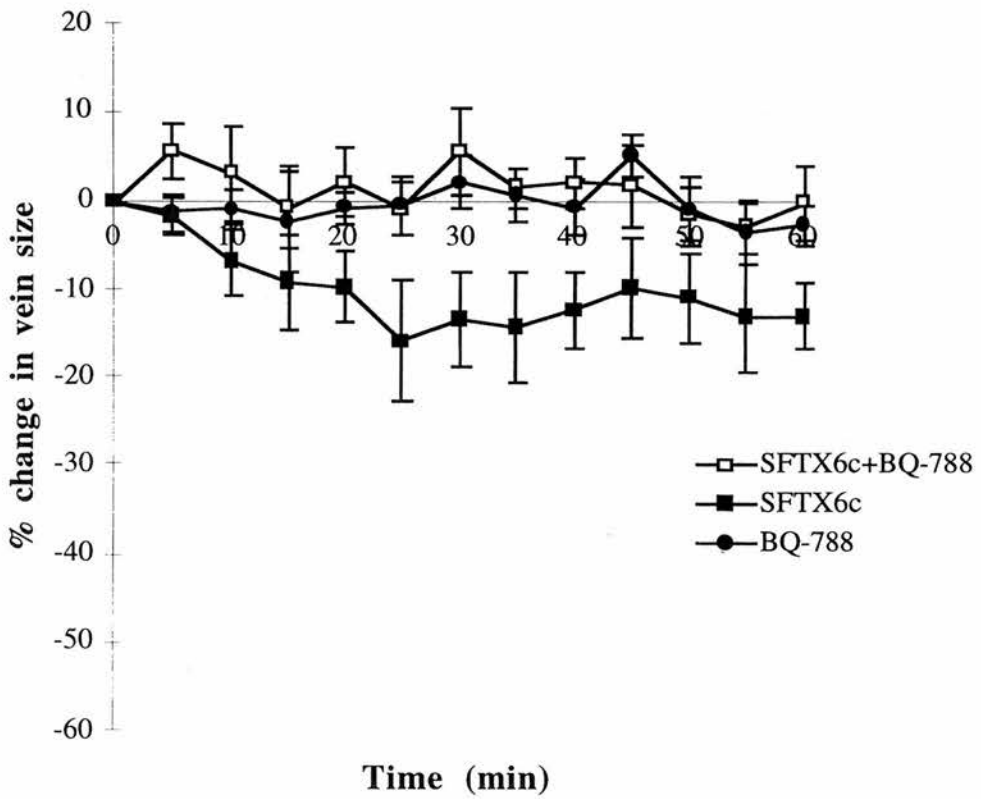
Figure 4.2 Response of hand vein diameter to local intravenous infusion of ET-1 (5 pmol/min) alone (closed circles) and during co-infusion with BQ-123 (1 nmol/min; open circles) or BQ-788 (1 nmol/min; open squares). Responses are expressed as mean % change in blood flow ratio ± SEM.



Study 3 Intra-venous sarafotoxin S6c and BQ-788

Hand vein diameter was significantly reduced following infusion of SFTX6c (13±4%, $p<0.001$). This constriction was abolished in the presence of BQ-788 (0±4%, $p=0.03$ vs SFTX6c alone, $p=0.9$ vs basal) (Figure 4.3). There was no significant change in vein size in response to BQ-788 alone (-3±2%, $p=0.9$ vs basal).

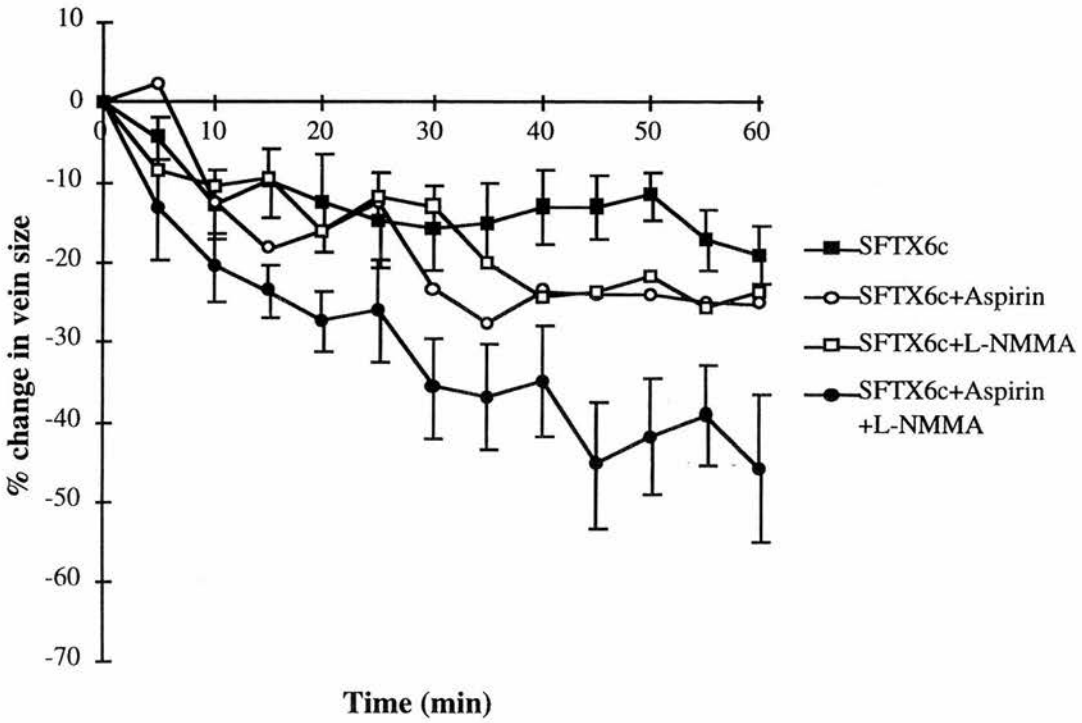
Figure 4.3 Response of hand vein diameter to local intravenous infusion of SFTX6c (5 pmol/min) alone (closed squares), BQ-788 (1 nmol/min) alone (Closed circles) and during co-infusion of SFTX6c and BQ-123 (open squares). Responses are expressed as mean % change in blood flow ratio ± SEM.



Study 4 Intra-venous sarafotoxin S6c and L-NMMA with Aspirin

SFTX6c caused significant venoconstriction (19±4%, p=0.003) which tended to increase after administration of aspirin (25±7%) and during co-infusion of L-NMMA (24±10%), but was not significantly different from sarafotoxin S6c alone (Figure 4.4). Venoconstriction to SFTX6c was substantially and significantly increased in the presence of aspirin and L-NMMA together (46±9%, p=0.0001; p=0.01 vs. S6c).

Figure 4.4 Response of hand vein diameter to local intravenous infusion of SFTX6c (5 pmol/min) alone (closed squares), during co-infusion of L-NMMA (100 nmol/min; open squares), following oral administration of Aspirin (600 mg; open circles) and during combined treatment with L-NMMA and Aspirin (closed squares). Responses are expressed as mean % change in blood flow ratio ± SEM.



4.4 Discussion

The results from this series of studies have confirmed the constrictor effects of the endothelin receptor agonists ET-1 and SFTX6c in dorsal hand veins *in vivo* (Haynes, et al., 1995; Strachan, et al., 2000). Venoconstriction to ET-1 was attenuated in the presence of the ET_A receptor selective antagonist BQ-123, but was not affected by co-infusion of the ET_B receptor selective antagonist BQ-788, suggesting that the ETA receptor is more important in mediating the constrictor effects of ET-1 than the ET_B receptor in healthy blood vessels.

Interestingly, the constriction to ET-1 was not completely blocked by BQ-123 at two dose levels. This could be simply due to incomplete blockade of the ET_A receptor with the doses chosen, however the concentrations achieved are estimated to be within the range sufficient to block the actions of ET-1 at the ET_A receptor. Inhibition of the response to ET-1 was greater with the higher dose of BQ-123, this could indicate that the higher dose was more effective producing a more complete blockade of the effects of the ET_A receptor or may result from non-selective effects of BQ-123 with the higher dose level. More importantly, incomplete blockade of the effects of ET-1 by BQ-123 could indicate a role for the ET_B receptor in the ET-1 response, either directly through ETB mediated constriction or indirectly by a cross talk mechanism (Mickley, et al., 1997; Seo, et al., 1994).

Venoconstriction to ET-1 was not affected by the selective ETB receptor antagonist BQ-788, similar effects have been described in animal studies (Mickley, et al., 1997; Seo, et al., 1994) and would seem to dispute a direct role for ETB receptor mediated constriction in ET-1 mediated venoconstriction. Interestingly, BQ-788 did not potentiate the response to ET-1 indicating that any increase in ET-1 concentrations caused by infusion of BQ-788, as a result of reduction in ETB receptor clearance of ET-1, did not lead to additional vasoconstriction mediated by unoccupied ET_A

receptors. This lack of potentiation could reflect a lack of available binding sites for increased circulating ET-1 following pseudo-irreversible binding of ET-1 to the ET_A receptor.

Selective ET_A receptor antagonism did not appear to affect the constriction mediated by SFTX6c, which contradicts the suggestion that the constrictor effects of ET_B receptor agonists result solely from displacement of ET-1, through reduced ET_B receptor mediated clearance, onto unoccupied ET_A receptors.

In Study 3 the selective ET_B receptor antagonist BQ-788 completely blocked the effects of SFTX6c showing that the current dose level of BQ-788 is sufficient to block the effects of the ET_B receptor *in vivo*. The fact that BQ-788 inhibits the response to SFTX6c *in vivo* further supports the use of SFTX6c as an ET_B receptor selective agonist (Strachan, et al., 2000). These data highlight the potential for both agents as tools for use in the investigation of ET_B mediated actions in health and cardiovascular disease.

BQ-788 had no effect on hand vein diameter when infused alone. In contrast, there was a small but significant reduction in vein diameter following infusion of BQ-123. This reduction in vein size could result from the actions of ET-1 at the unoccupied vascular smooth muscle ET_B receptors. It is unlikely, although possible, that this small reduction in vein size with BQ-123 infusion would have a significant effect on the results during co-infusion with the endothelin receptor agonists described. Combined administration of BQ-123 and BQ-788 alone and during co-infusion of ET-1 would help clarify further the functional significance of ET_B receptor mediated constriction.

In Study 4 the role of the endothelium-derived dilators nitric oxide and prostacyclin in the venoconstrictor effects of SFTX6c were investigated. The constrictor effects of

SFTX6c were confirmed, and, as seen previously, were substantially less than those of ET-1 shown in earlier studies. These findings support the hypothesis that ET_B receptors contribute to, but do not wholly account for, ET-1 mediated venoconstriction. In addition, venoconstriction to sarafotoxin S6c is significantly and substantially increased when generation of both nitric oxide and dilator prostanoids, most likely prostacyclin, are blocked. Combined inhibition of nitric oxide and prostacyclin appeared to produce a greater effect on venoconstriction to sarafotoxin S6c than the addition of the individual effects of inhibition of generation of either nitric oxide or prostacyclin alone. This may reflect a capacity for the endothelium to compensate for inhibition of one dilator mediator by increased production of another. Earlier studies have shown that responses to ET-1 are potentiated by aspirin administration but not by co-infusion of L-NMMA (Haynes and Webb, 1993). It would appear from these results with SFTX6c that the endothelial ET_B receptor modulates the constrictor effects produced by stimulation of the vascular smooth muscle ET_B receptors, through generation of vasodilator substances by the endothelium. However, in these experiments, the vasoconstrictor action predominates. In diseases such as chronic heart failure, where endothelium-dependent vasodilatation is impaired, venoconstrictor effects of ET-1 may be enhanced as a result of unopposed vasoconstrictor effects mediated by both ET_A and ET_B receptors on vascular smooth muscle. Indeed local vasoconstriction to SFTX6c is enhanced in heart failure patients (Love, et al., 1996) and may indicate upregulation of ET_B receptor mediated constriction in heart failure.

Given that the overall effect of combined stimulation of the vascular smooth muscle and endothelial cell ET_B receptors is venoconstriction, and incomplete attenuation of ET-1 mediated constriction following ET_A receptor antagonism in the hand vein, it may be necessary to block both ET_A and ET_B receptors, in order to completely block venoconstriction to ET-1. Drugs which would act specifically on the vascular smooth

muscle receptors (Warner, et al., 1993) may be more effective vasodilators, since they would fully block ET-1 mediated vasoconstriction, while allowing the potentially desirable effects of the endothelial ET_B receptors to be preserved.

Chapter 5

Local vasoconstrictor and vasodilator effects of endothelin-B receptor agonists in forearm resistance vessels *in vivo*.

Constriction to ETB receptor agonists, BQ-3020 and sarafotoxin S6c, in human resistance and capacitance vessels *in vivo*. Strachan FE, Crockett, TR, Mills NM, Gray GA, Webb DJ. Br J Clin Pharmacol 2000;50:27-30.

5.1 Introduction

As previously discussed, the powerful vasoconstrictor and vasopressor effects of endothelin-1 (ET-1) (Clarke, et al., 1989; Yanagisawa, et al., 1988) are predominantly mediated via the ET-1 selective, vascular smooth muscle cell ET_A receptor (Arai, et al., 1990). ET_B receptors have also been described on vascular smooth muscle cells (Davenport, et al., 1993) and may contribute to the vasoconstrictor effects of ET-1 (Clozel, et al., 1992). In addition to the vasoconstrictor effects of endothelin receptor agonists, transient hypotension, preceding sustained pressor effects, has been noted following bolus doses of the endothelin peptides. This effect is most noticable with ET-3 (Inoue, et al., 1989) and is thought to be caused by generation of endothelium-derived dilators nitric oxide and prostacyclin, mediated by endothelial cell ET_B receptors. However, although likely to be mediated by the actions of the endothelial ET_B receptor, no single mediator of this transient depressor effect has been proposed. Inhibition of nitric oxide synthase (Fozard and Part, 1992) and cyclo-oxygenase (Filep, et al., 1993) have been shown to attenuate this depressor response, although the effects of cyclo-oxygenase were not found to be significant by other investigators (DeNucci, et al., 1988).

Chapter 3 and Chapter 4 describe the local constrictor effects of ET_B receptor selective agonists in hand veins *in vivo* (Haynes, et al., 1995; Strachan, et al., 2000). Similarily, local vasoconstriction in response to intra-arterial infusion of the non-selective endothelin receptor agonist ET-1 (Clarke, et al., 1989; Haynes, et al., 1995) and the ET_B receptor selective agonist ET-3 (Haynes, et al., 1995) has also been described in the forearm resistance vessels. As in the hand vein studies, the response to ET-3, although significant, was less than that to ET-1. Transient vasodilatation followed by local vasoconstriction has previously been demonstrated in the forearm resistance vessels *in vivo* in response to high dose infusion of the endogenous ET_B

receptor agonist ET-3 (Haynes, et al., 1995). In contrast, this effect is not evident with low dose infusion of ET-1 in the forearm resistance vessels (Haynes, et al., 1995). In view of the relatively high doses required to effect this vasodilator effect it is likely that vasodilatation to endothelin receptor agonists represents a pharmacological rather than a physiological phenomenon. However, using this response to demonstrate vasodilatation *in vivo* allows investigation of the mechanisms involved in mediating this dilator effect.

In order to investigate the effects of ET_B receptor stimulation in the arterial circulation further the effects of low dose intra-arterial infusion of two structurally distinct ET_B receptor agonists, SFTX6c and BQ-3020, on forearm blood flow was assessed in Study 1. In Study 2, ET_B receptor mediated vasodilatation was investigated following high dose infusion of SFTX6c. The role of endothelium-derived dilators in this response was investigated using aspirin as an inhibitor of dilator prostaglandins (Heavey, et al., 1985) and L-NMMA to inhibit generation of nitric oxide (Vallance, et al., 1989) and BQ-788 was used as a selective ET_B antagonist.

5.2 Methods

5.2.1 Subjects

Eight healthy male subjects within the age range of 18 - 60 years, were recruited to Study 1 and six were recruited to Study 2, under the standard conditions described in Chapter 2.

5.2.2 Drugs

Study 1

Sarafotoxin S6c (5 pmol/min) and BQ-3020 (50 pmol/min) were administered by continuous infusion for 90 min.

Study 2

Sarafotoxin S6c (60 pmol/min) was administered by continuous infusion for 5 min. BQ-788 (1 nmol/min), L-NMMA (4 μmol/min) or saline was administered for 60 min prior to and for 30 min following SFTX6c infusion. Aspirin (600mg) was administered orally 30 min before infusion of SFTX6c.

Study 2 was extended, in 3 subjects, to include assessment of the response to SFTX6c in the presence of noradrenaline. Sarafotoxin S6c (60 pmol/min) was administered by continuous infusion for 5 min. Noradrenaline (160 pmol/min) or saline was administered for 60 min prior to and for 30 min following SFTX6c infusion.

In all studies, saline was infused for 30 min before infusion of the study agent, the total infusion rate was kept constant at 1 ml/min.

Intra-arterial Administration

The brachial artery of the non-dominant arm was cannulated under local anaesthetic (1% Lignocaine; Astra Pharmaceuticals, Kings Langley, England) with a 27 SWG steel cannula (Cooper's Needle Works, Birmingham, England).

5.2.3 Measurements

Forearm Blood Flow

The response to intra-arterial infusion was assessed by measurement of forearm blood flow in the infused and non-infused forearm by venous occlusion plethysmography as described in Chapter 2.

In Study 1, forearm blood flow recordings were made at 10 min intervals throughout the intra-arterial infusion. In Study 2, forearm blood flow was recorded continuously

for 13 min from the 3 min before the start of the SFTX6c infusion, until 5 min after the end of the SFTX6c infusion. At all other times during the intra-arterial infusions, forearm blood flow recordings were made at 10 min intervals.

Blood Pressure and Heart Rate

Blood pressure and heart rate were measured in the non-infused arm using a well-validated semiautomated non-invasive method at 30 min intervals throughout the infusions as described in Chapter 2.

5.2.4 Study Design

Studies were performed single blind, with the experimental subjects, but not the investigators, blinded to the peptide and dose administered in each study. Oral administration of aspirin was unblinded.

Study 1 Intra-arterial sarafotoxin S6c and BQ-3020

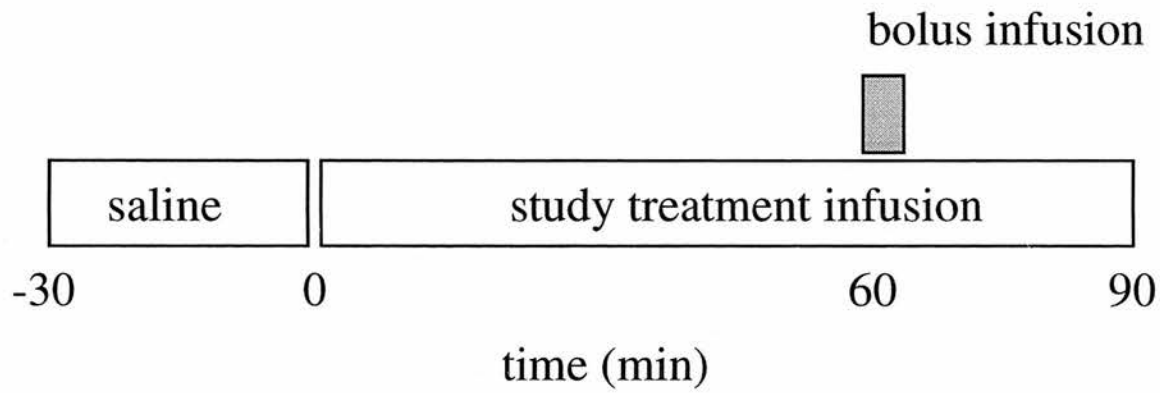
The local effects of intra-venous (see Chapter 3) and intra-arterial infusion of BQ-3020 and SFTX6c were investigated in eight healthy male subjects in a single-blind, randomised, 4 way crossover study. Subjects attended for 4 visits, each separated by at least one week. On separate occasions, in a random order, each subject received either intra-venous infusion of SFTX6c (5 pmol/min) or BQ-3020 (50 pmol/min) for 90 min, or intra-arterial infusion of SFTX6c (5 pmol/min) or BQ-3020 (50 pmol/min), the intra-venous studies are discussed in Chapter 3.

Study 2 High dose infusion of sarafotoxin S6c and the effects of BQ-788, L-NMMA and Aspirin

The local effects of intra-arterial infusion of SFTX6c were investigated in six healthy male subjects in a single-blind, randomised, 5 way crossover study. Subjects attended for 5 visits, each separated by at least one week. On separate occasions, in a random order, SFTX6c (60 pmol/min) for 5 min was administered by intra-arterial infusion, during co-infusion with either BQ-788 (1 nmol/min), L-NMMA (4 μmol/min) or saline, or following oral administration of aspirin (600 mg). BQ-788, L-NMMA and saline were infused for 60 min before and for 30 min after the start of infusion of SFTX6c according to the study randomisation (Figure 5.1). On one of the visits in random order, BQ-788 (1 nmol/min) was administered alone by intra-arterial infusion for 90 min. On this visit, saline was administered for 5 min, in the same manner as SFTX6c on all other studies, in order to provide a control for the method of bolus administration and the measurement technique.

In order to investigate the response to SFTX6c in the presence of a constrictor agent acting by a different mechanism than BQ-788 or L-NMMA, study 2 was extended to investigate the response to intra-arterial infusion of SFTX6c during co-infusion of noradrenaline, in three healthy male subjects in a single-blind, randomised, 2 way crossover study. On separate occasions, each separated by at least one week, in a random order, SFTX6c (60 pmol/min) for 5 min was administered by intra-arterial infusion, during co-infusion with either noradrenaline (160 pmol/min) or saline. Noradrenaline and saline were infused for 60 min before and for 30 min after the start of infusion of SFTX6c according to the study randomisation.

Figure 5.1 Schematic representation of the study protocol for Study 2: BQ-788 (1 nmol/min), L-NMMA (4 μmol/min), noradrenaline (160 pmol/min) or saline were administered as study treatments, SFTX6c (60 pmol/min) or saline was administered for 5 min as a bolus infusion 60 min after the start of the study treatment infusion. On one occasion aspirin (600 mg) was administered orally before bolus infusion of SFTX6c.



5.2.5 Statistical Analysis

Forearm blood flow

Plethysmographic data listings were extracted from data files and forearm blood flows calculated for individual venous occlusion cuff inflations using a template spreadsheet (Excel 5.0; Microsoft Ltd, Wokingham, UK), as described in Chapter 2. Forearm blood flow results are expressed as the percentage change from baseline in the ratio of blood flow between the infused and non-infused arms.

Study 1

All results are expressed as mean ± standard error of the mean (SEM) at 90 min. Responses were examined by repeated-measures analysis of variance (ANOVA) (Excel 5.0, Microsoft Ltd, Wokingham, UK).

Study 2

The response to the bolus infusion is expressed as the % change from blood flow measured immediately before the start of the SFTX6c infusion. Results are expressed as mean ± standard error of the mean (SEM) at 2 min and 30 min after SFTX6c infusion. The forearm blood flow responses, from 0 - 5 min and 6 - 30 min, were examined by repeated-measures analysis of variance (ANOVA) (Excel 5.0, Microsoft Ltd, Wokingham, UK).

In both studies, heart rate and baseline measurements were compared using the Student's paired *t*-test (Excel 5.0, Microsoft Ltd, Wokingham, UK). For all comparisons, statistical significance was accepted at the 5% level.

5.3 Results

Eight healthy male subjects (age range 20 - 28 years) completed Study 1 and six healthy male subjects (age range 20 - 43 years) completed Study 2. There was no significant difference between baseline measurements on each of the study visits. There was no significant change in forearm blood flow in the non-infused arm at the end of the intra-arterial infusion, or in blood pressure and heart rate in the non-infused arm at the end of each infusion, confirming that any drug effects were confined to the infused arm (Table 5.1, Study 1; Table 5.2, Study 2).

Table 5.1 Mean arterial pressure (MAP), heart rate (HR), forearm blood flow (FBF) at baseline and at 90 min following the start of each infusion. Values are mean±SEM.

	BQ-3020 (50pmol)	**SFTX6c** (5pmol)
MAP (mmHg)		
Basal	93±3	86±2
90 Mins	96±5	92±3
HR (bpm)		
Basal	56±3	54±2
90 Mins	56±2	54±3
FBF (ml/100ml/min)		
Control Arm		
Basal	4.0±0.8	4.4±1.0
90 min	5.0±1.4	5.2±1.0
Infused Arm		
Basal	4.6±1.0	3.9±0.7
90 min	3.6±0.7	3.2±0.1

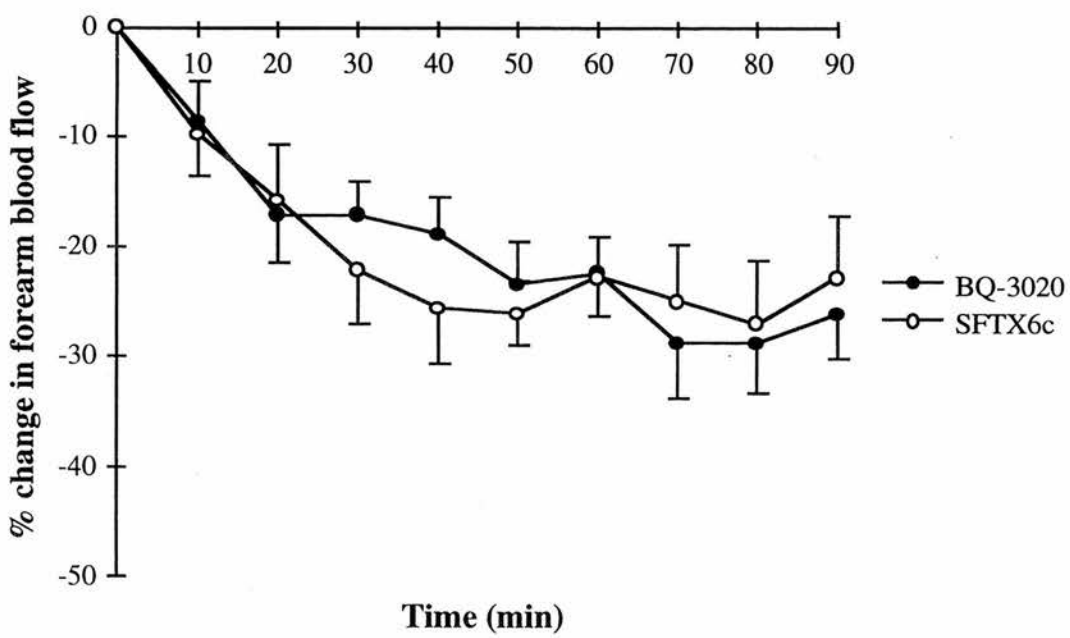
Table 5.2 Mean arterial pressure (MAP), heart rate (HR), forearm blood flow (FBF) at baseline and at the end of infusions (90 min).

	SFTX6c	**SFTX6c + BQ-788**	**SFTX6c + L-NMMA**	**SFTX6c + Aspirin**	**BQ-788**
MAP (mmHg)					
Basal	89±3	93±3	88±3	92±4	91±5
90 min	90±2	92±2	88±4	92±3	94±5
HR (bpm)					
Basal	62±6	58±4	59±2	54±1	56±3
90 min	61±6	58±3	58±2	56±3	61±4
FBF (ml/100ml/min)					
Control Arm					
Basal	2.8±0.3	3.6±0.8	3.7±0.4	2.9±0.4	3.6±0.6
90 min	3.8±0.7	4.5±0.6	3.6±0.3	3.2±0.6	3.8±0.4
Infused Arm					
Basal	3.2±0.3	3.9±1.0	4.8±0.7	3.3±0.5	4.0±0.9
90 min	3.5±0.5	3.4±0.9	2.3±0.4	2.9±0.3	3.6±0.4

Study 1 Intra-arterial sarafotoxin S6c and BQ-3020

Sarafotoxin S6c (5 pmol/min) caused a significant reduction in the ratio of forearm blood flow (-25±7%; $p<0.001$), indicating vasoconstriction in the infused arm (Figure 5.2). Similarly, BQ-3020 (50 pmol/min) caused significant vasoconstriction (-27±7%; $p<0.001$) (Figure 5.1). The response to both SFTX6c and BQ-3020 was slow in onset and reached a maximum after ~60 min. There was no significant difference between the forearm blood flow responses to SFTX6c and BQ-3020 ($p=0.5$).

Figure 5.2 Response of forearm blood flow to local intra-arterial infusion of SFTX6c (5 pmol/min; open circles) and BQ-3020 (50 pmol/min; closed circles). Responses are expressed as mean % change in blood flow ratio ± SEM. There was no significant difference ($p = 0.3$) between the responses to SFTX6c and BQ-3020, n=8.



Study 2 High dose infusion of sarafotoxin S6c and the effects of BQ-788, L-NMMA and Aspirin

SFTX6c alone caused a transient vasodilatation (22±8%; p<0.001 vs basal), with vasoconstriction developing after 5 min and continuing until 30 min after the start of the SFTX6c infusion (-20±4%, p<0.001 vs basal) (Figure 5.3). L-NMMA and BQ-788 completely abolished the vasodilatation at 2 min (-4±4%; -8±4%, respectively; p<0.001 vs SFTX6c for both) (Figure 5.4 and 5.5, respectively). In contrast, aspirin did not affect the vasodilator response (18±9%; p=0.6 vs SFTX6c) (Figure 5.5).

The vasoconstriction to SFTX6c at 30 min (-20±4%) was evident with all treatments (Aspirin, -21±3%; BQ-788, -26±6%; L-NMMA, -13±4%; p<0.001 for all). Although there was no significant difference in the response to SFTX6c between 6-30 min in the presence of Aspirin or L-NMMA (p=0.8 vs SFTX6c for both), there was a significant difference in the presence of BQ-788 (p<0.001, 6-30 min; p=0.05, 10-30 min vs SFTX6c).

As expected L-NMMA caused significant vasoconstriction before infusion of SFTX6c (L-NMMA: -47±2%, p<0.001) (Figure 5.6). Infusion of BQ-788 alone also caused vasoconstriction (-15±3%, p<0.001 at 60 min) (Figure 5.6). Saline was infused during the BQ-788 alone visit in the same manner as the SFTX6c infusion in all other treatments, when the data were represented relative to the timing of the 'bolus' infusion there was no dilatation evident at 2 min (0.1±1.4%, p=0.2 vs basal) or constriction at 30 min (-5±5%, p=0.4 vs basal) (Figure 5.3).

In the extension of Study 2, noradrenaline caused vasoconstriction prior to infusion of SFTX6c (-46±14% at 60 min). SFTX6c caused a transient dilatation in the presence of noradrenaline (15±3% at 2 min after bolus infusion) with constriction developing after 3 min (-18±8% at 30 min) (Figure 5.7).

Figure 5.3 Response of forearm blood flow to local intra-arterial infusion of SFTX6c (60 pmol/min for 5 min; closed circles) and BQ-788 (1 nmol/min; open circles, saline was infused for 5 min in the same manner as SFTX6c). SFTX6c caused transient vasodilatation followed by vasoconstriction. Bolus infusion of saline had no effect on forearm blood flow. Responses are expressed as mean % change, from pre-bolus infusion, in blood flow ratio ± SEM.

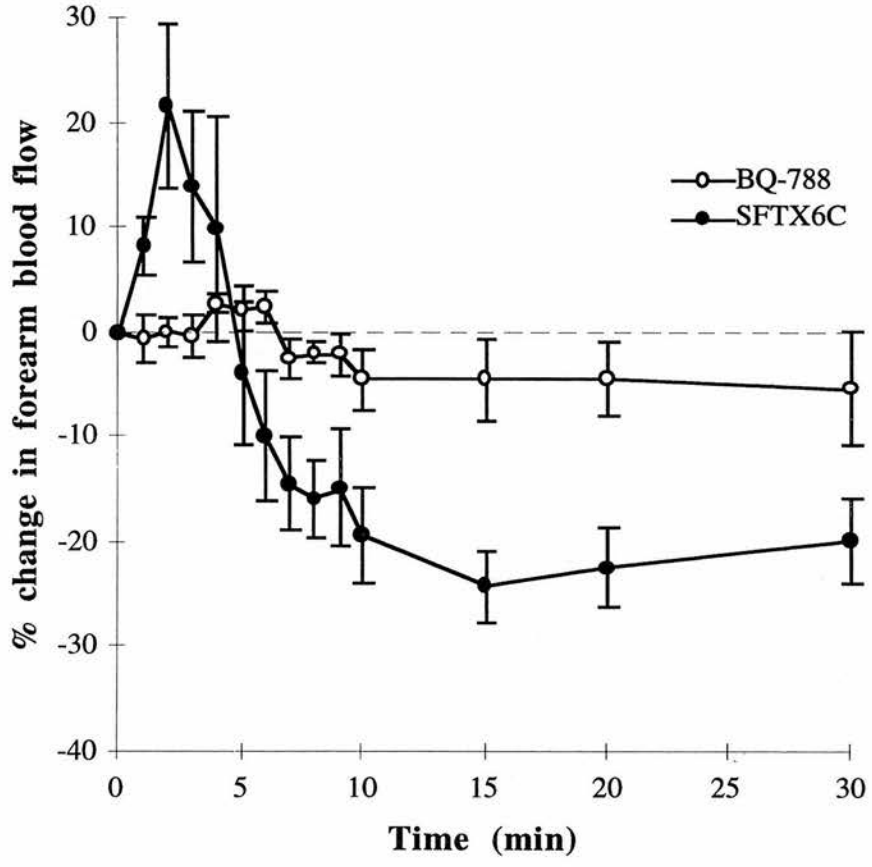


Figure 5.4 Response of forearm blood flow to local intra-arterial infusion of SFTX6c (60 pmol/min for 5 min; closed circles) alone and during co-infusion of BQ-788 (1 nmol/min; open circles). SFTX6c caused transient vasodilatation followed by vasoconstriction. BQ-788 completely blocked the vasodilator response to SFTX6c and potentiated the development of the vasoconstrictor response. Responses are expressed as mean % change, from pre-bolus infusion, in blood flow ratio ± SEM.

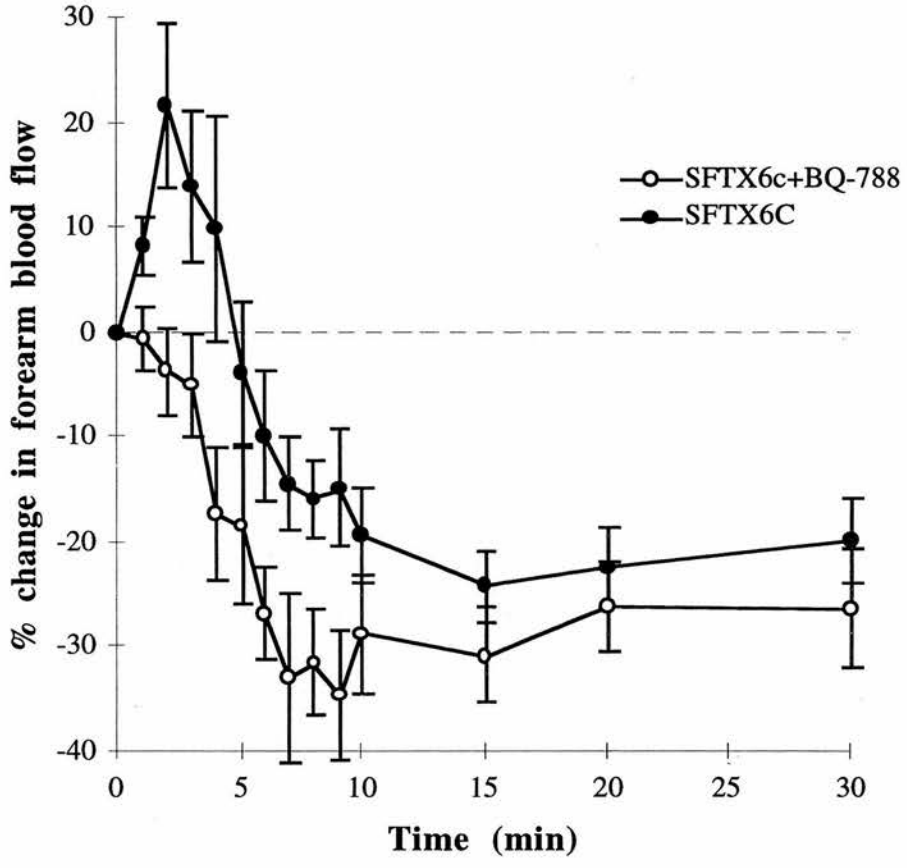


Figure 5.5 Response of forearm blood flow to local intra-arterial infusion of SFTX6c (60 pmol/min for 5 min; closed circles) alone, during co-infusion of L-NMMA (4 μmol/min; open circles) and following oral administration of Aspirin (600 mg; open squares). SFTX6c caused transient vasodilatation followed by vasoconstriction. L-NMMA completely blocked the vasodilator response to SFTX6c but had no effect on the vasoconstrictor response. Oral administration of aspirin had no effect on the vasodilat or vasoconstrictor response to SFTX6c. Responses are expressed as mean % change, from pre-bolus infusion, in blood flow ratio ± SEM.

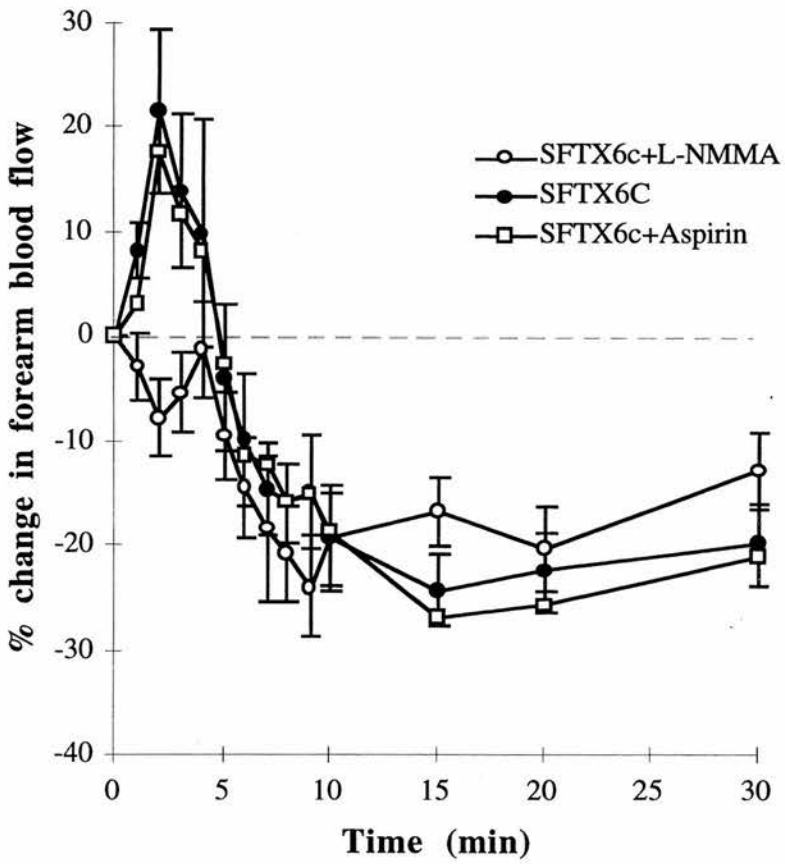


Figure 5.6 Response of forearm blood flow to local intra-arterial infusion of BQ-788 (1 nmol/min; open circles) and L-NMMA (4 μmol/min; closed circles) in the 60 min before the bolus infusion. Responses are expressed as mean % change, from pre-bolus infusion, in blood flow ratio ± SEM.

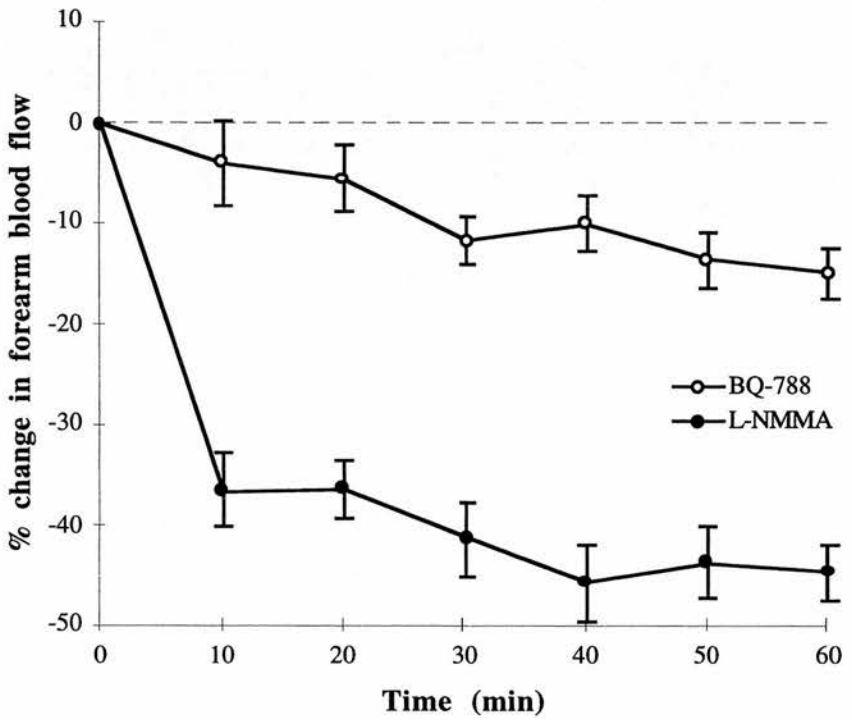
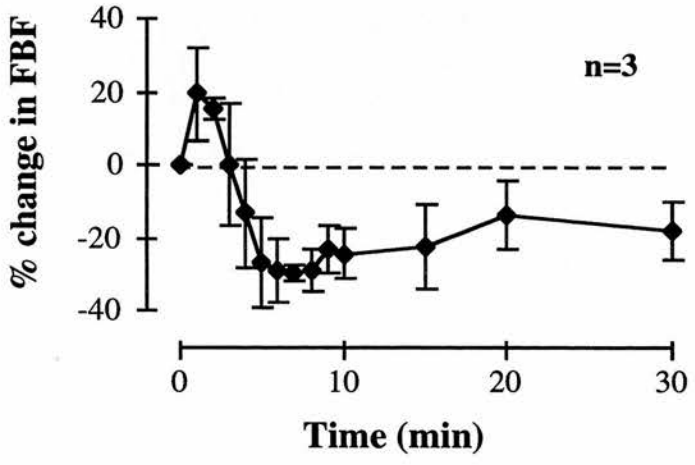


Figure 5.7 Response of forearm blood flow to local intra-arterial infusion of SFTX6c (60 pmol/min for 5 min) during co-infusion of noradrenaline (160 pmol/min) in 3 subjects. SFTX6c caused transient vasodilatation followed by vasoconstriction during co-infusion of noradrenaline. Responses are expressed as mean % change, from pre-bolus infusion, in blood flow ratio ± SEM.



5.4 Discussion

The results of Study 1 have confirmed that infusion of a locally active dose of the ET_B receptor agonist SFTX6c causes vasoconstriction (Haynes, et al., 1995) in healthy blood vessels *in vivo*. We have also extended these observations to show, for the first time, that similar effects are seen with a structurally distinct ET_B receptor selective agonist, BQ-3020, with close homology to ET-1. The fact that constriction also occurs in response to BQ-3020 provides evidence that the effects previously described with SFTX6c are not idiosyncratic to this peptide and may be representative of endogenous binding of ET-1 to the ET_B receptor. Therefore, concerns over signalling pathway differences between SFTX6c and endothelin peptide binding (Shraga-Levine, et al., 1994; Sokolovsky, 1995) may not be functionally important. In contrast to the results in the hand veins, described in Chapter 3, there was no difference in the response to SFTX6c and BQ-3020 in the forearm resistance vessels.

The results from Study 2 demonstrate that a bolus dose of the selective ET_B agonist SFTX6c causes transient vasodilatation followed by vasoconstriction *in vivo* in human forearm resistance vessels, consistent with previous results with the endogenous ET_B receptor agonist ET-3 (Haynes, et al., 1995). Inhibition of the vasodilator response by both BQ-788 and L-NMMA, but not aspirin, suggests that this vasodilatation is mediated by the ET_B receptor through increased NO generation. Interestingly, the constriction to SFTX6c was still evident with each of the study treatments. However, there was a significant difference between the constriction with SFTX6c alone and in the presence of BQ-788. Although the constriction was still evident in the presence of BQ-788, the difference appeared to be in the development of the response, perhaps due to inhibition of the dilator effects of the endothelial ET_B receptor potentiating the constrictor effects of the vascular smooth muscle receptors. Infusion of BQ-788 alone caused vasoconstriction, which could indicate that the balance of effects at the ET_B

receptor favour vasodilatation, this response is discussed in more detail in Chapters 6 and 10.

Although the vasodilator response to SFTX6c was completely blocked in the presence of the selective ET_B receptor antagonist BQ-788, the constrictor response was not reduced, in fact it seemed to develop more quickly during co-infusion with BQ-788. These results could indicate that the constrictor response following the transient vasodilatation is not directly mediated by the vascular smooth muscle cell ET_B receptor. It is possible that this constrictor response is mediated by the ET_A receptor either through reduced clearance of ET-1 via ET_B receptors following SFTX6c infusion or as a result of ET_B receptor desensitisation following the high dose infusion of SFTX6c.

Alternatively, although SFTX6c mediated constriction in the hand vein was completely blocked by the same dose of BQ-788 (Chapter 4, Study 3), the dose of BQ-788 may not have been sufficient to block effects of SFTX6c at the endothelial and vascular smooth muscle cell ET_B receptors in the forearm resistance vessels in the current study. It has also been suggested that some ET_B receptor antagonists, including BQ-788, act preferentially at the endothelial ET_B receptors and are less potent at the vascular smooth muscle ET_B receptors (Schroeder, et al., 1998), this could account for the lack of effect of BQ-788 on SFTX6c mediated vasoconstriction in the current study. It is possible that the predominant effects of intra-luminal infusion of BQ-788 selectively affect the endothelial ET_B receptor because the drug has better access to the endothelial than to the smooth muscle receptors.

It is important to recognise that infusion of BQ-788 alone causes significant vasoconstriction and so it may not be possible to demonstrate attenuation of the constriction to SFTX6c on the background of the BQ-788 effect. Interpretation of the

results of co-infusion studies is difficult due to the individual effects of each agent on vascular tone.

Intra-arterial infusion of L-NMMA also causes significant vasoconstriction due to blockade of endogenous nitric oxide generation (Vallance, et al., 1989). Therefore, vasodilatation to SFTX6c may be difficult to demonstrate on the background of this substantial forearm vasoconstriction. In order to investigate whether the lack of effect was due to the inhibition of nitric oxide generation caused by L-NMMA or simply due to the vasoconstriction present in the forearm a follow-on study in 3 subjects assessing the response to SFTX6c infusion following preconstriction with noradrenaline was performed. The results of this small study demonstrate vasodilatation following administration of SFTX6c in the presence of noradrenaline mediated vasoconstriction, supporting the hypothesis that the blockade of the dilator response in the presence of L-NMMA results from nitric oxide inhibition and not merely as an additive effect of the L-NMMA and the SFTX6c response. The apparent lack of involvement of dilator prostaglandins in the vasodilator response to SFTX6c is consistent with previous studies (DeNucci, et al., 1988) but in contrast with others (Filep, et al., 1993).

We have demonstrated local vasoconstriction in healthy blood vessels *in vivo* in response to locally active infusions of two structurally distinct ET_B receptor selective agonists. In addition we have confirmed that high dose infusion of ETB receptor selective agonists causes vasodilatation and demonstrated that this vasodilatation is mediated via the ETB receptor through increased nitric oxide generation. The importance of ETB mediated vasodilatation was demonstrated by the vasoconstrictor response to administration of the ETB receptor selective antagonist BQ-788. This response will be discussed in future chapters and the relative benefits of selective and non-selective ET receptor antagonists investigated.

Chapter 6

The local vasodilator effects of selective ET_A and combined ET_A/ET_B receptor antagonism in healthy volunteers

Endothelin-A receptor antagonist mediated vasodilatation is attenuated by inhibition of nitric oxide synthesis and by endothelin-B receptor blockade. Verhaar MC, Strachan FE, Newby DE, Cruden NL, Koomans HA, Rabelink TJ, Webb DJ. Circulation 1998;97:752-756.

6.1 Introduction

As discussed in earlier chapters, the contribution of the vascular ET_B receptor to the recognised endogenous ET-1 mediated constrictor tone depends on the balance between the ET_B receptor mediated effects of vasodilatation, vasoconstriction and ET-1 clearance. The contribution of the ET_A and ET_B receptors to ET-1 mediated vascular tone is of mechanistic importance and, potentially, of major therapeutic relevance in the development of endothelin receptor antagonists. A number of selective ET_A and non-selective ET_A/ET_B receptor antagonists are currently in clinical development as vasodilator agents (Strachan and Webb, 1998; Weber, et al., 1996). Although both selective and non-selective endothelin receptor antagonists have demonstrated vasodilator effects in healthy subjects (Haynes, et al., 1996) in patients with heart failure (Kiowski, et al., 1995) and in patients with hypertension (Cardillo, et al., 1999; Ferro, et al., 1996; Taddei, et al., 1999), the question of whether selective ET_A or combined ET_A/ET_B receptor antagonism will be of more benefit as vasodilator therapy remains to be clarified.

Chapter 5 describes local vasoconstriction in response to infusion of the ET_B receptor selective antagonist BQ-788. In order to investigate the role of the ET_A and ET_B receptor subtypes and their possible interactions in mediating the vasodilator response to selective ET_A receptor antagonism (Haynes and Webb, 1994), the effects of locally active doses of the ET_A receptor selective antagonist BQ-123, the ET_B receptor selective antagonist BQ-788 and their combined effects in forearm resistance vessels were assessed in the current study.

6.2 Methods

6.2.1 Subjects

Sixteen healthy male subjects within the age range of 18-60 years were recruited to the study, under the standard conditions listed in chapter 2.

6.2.2 Drugs

BQ-123 (10 nmol/min) and BQ-788 (1 nmol/min) were administered by continuous infusion for 120 min. Saline was infused for 30 min before infusion of the study agent. The total infusion rate was kept constant at 1 ml/min.

Local forearm vasodilatation to intra-arterial infusion of BQ-123 (100 nmol/min) has been demonstrated previously (Haynes and Webb, 1994). A ten-fold lower dose of BQ-123 (10 nmol/min) was used in the current study because it has been shown more recently that this dose causes vasodilatation of equal magnitude to that seen with the higher dose (Ferro, et al., 1996).

6.2.3 Measurements

Forearm Blood Flow

The response to intra-arterial infusion was assessed by measurement of forearm blood flow in the infused and non-infused forearm by venous occlusion plethysmography as described in Chapter 2.

Blood Pressure and Heart Rate

Blood pressure and heart rate were measured in the non-infused arm using a well-validated semi-automated non-invasive method at 30 min intervals throughout the infusions as described in Chapter 2.

6.2.4 Study design

The study was performed single blind, with the experimental subjects, but not the investigators, blinded to the peptide and dose administered in each study.

On 2 separate study days in random order, in eight subjects, the ET_A receptor antagonist, BQ-123, was infused for 120 min alone, or during co-infusion of BQ-788, also for 120 min. On a separate occasion, BQ-788 was infused alone in eight subjects (two of whom also participated in the earlier parts of the study). Each study day was separated by at least a week.

6.2.5 Analysis

Forearm blood flow

Plethysmographic data listings were extracted from data files and forearm blood flows calculated for individual venous occlusion cuff inflations using a template spreadsheet (Excel 5.0; Microsoft Ltd, Wokingham, UK), as described in Chapter 2. Forearm blood flow results are expressed as the percentage change from baseline in the ratio of blood flow between the infused and non-infused arms.

All results are expressed as mean ± standard error of the mean (SEM) at 120 min. Blood pressure, heart rate and baseline measurements were compared using the Student's paired *t*-test.

Responses were examined by repeated measures analysis of variance (ANOVA). Statistical significance was taken at the 5% level and analysis was performed using an Excel data analysis package (Excel 5.0; Microsoft Ltd, Wokingham, UK).

6.3 Results

Fourteen healthy men (age range 20-43 years) completed the study, 2 subjects participated in the first (BQ-123 alone or in combination with BQ-788) and the second (BQ-788 alone) part of the study. There were no significant changes in baseline hemodynamics between phases of each study (Table 6.1), and no change in blood pressure, or blood flow in the non-infused forearm, during the course of the studies.

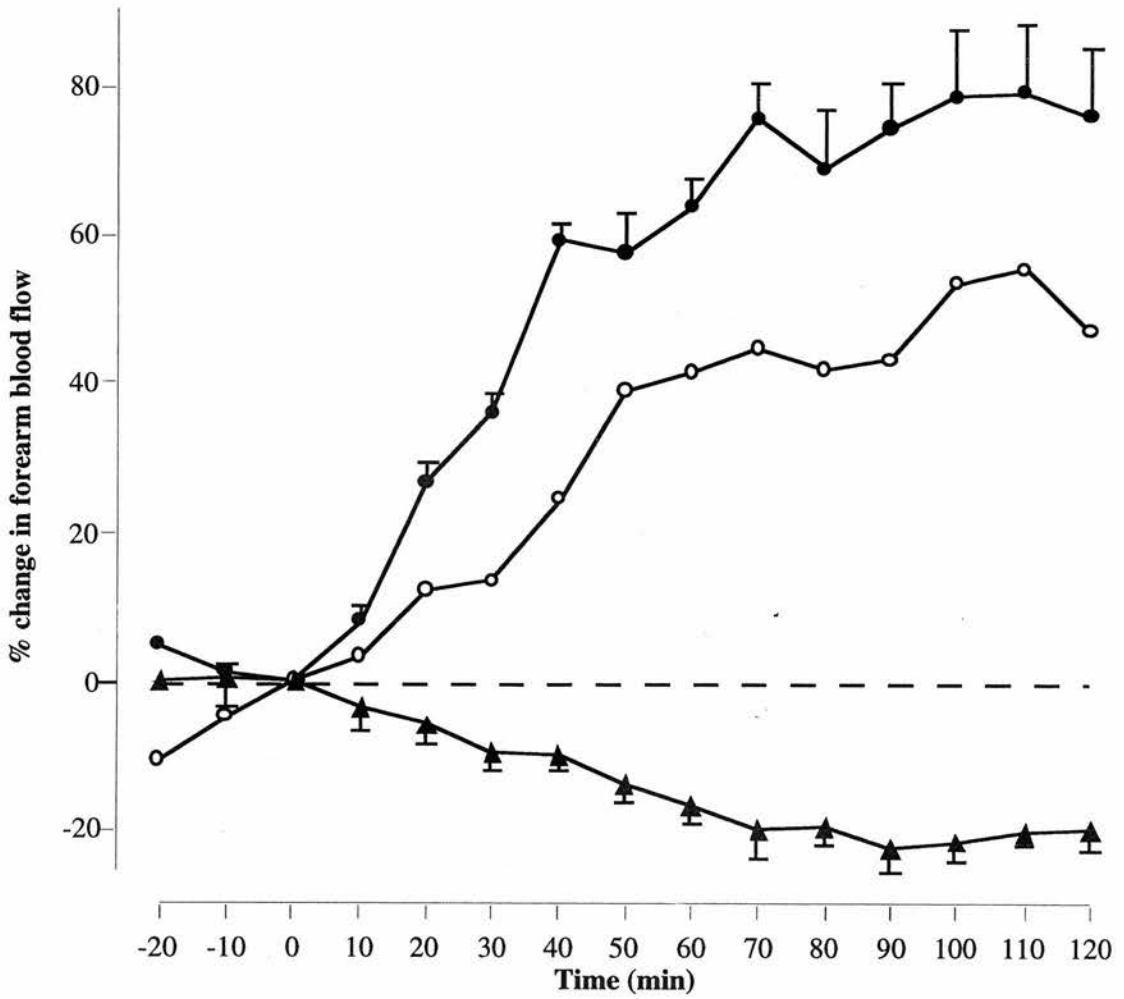
Forearm blood flow

Both BQ-123 alone and co-administration of BQ-123 and BQ-788 caused progressive vasodilatation ($p<0.001$) which appeared to plateau at 60 min (Figure 6.1). The vasodilatation to BQ-123 alone was significantly greater than during co-infusion with BQ-788 (76±13% vs. 47±14% at 120 min, $p<0.001$). BQ-788 alone caused a small but consistent reduction in forearm blood flow (20±3% at 120 min, $p<0.001$) (Figure 6.1).

Table 6.1 Mean arterial pressure (MAP) and forearm blood flow (FBF) predose and at 120 min afterintra-arterial infusion of BQ-123, BQ-788 or co-infusion of BQ-123 and BQ-788. Values are mean ± SEM.

	BQ-123 (10 nmol/min)	**BQ-123 +BQ-788**	**BQ-788** (1 nmol/min)
Forearm Blood Flow (ml/100ml/min)			
Infused arm	3.6±0.6	4.5±1.0	4.5±0.7
Control arm	3.4±0.6	3.3±0.8	3.1±0.4
Mean arterial pressure (mmHg)	83±5	90±3	93±4

Figure 6.1 Eight subjects received brachial artery infusion of BQ-123 (10 nmol/min) alone, closed circles; BQ-788 (1 nmol/min) alone, closed triangles; BQ-123 (10 nmol/min) co-infused with BQ-788 (1 nmol/min), open circles. Slow onset vasodilatation occurred in response to BQ-123, this response was attenuated during co-infusion of BQ-788. BQ-788 infusion alone caused a small but significant vasoconstriction.



6.4 Discussion

Earlier results demonstrating the importance of endogenous ET-1 in the mediation of vascular tone (Ferro, et al., 1996; Haynes and Webb, 1994) have been confirmed by the demonstration of slow onset forearm vasodilatation to local arterial infusion of the selective ET_A receptor antagonist BQ-123. The observation that the vasodilator response to combined ET_A and ET_B receptor antagonism was significantly less than to selective ET_A receptor antagonism alone could reflect the presence of an endogenous ET_B mediated vasodilator tone. This is further supported by the local vasoconstrictor effect of ET_B receptor antagonism in the forearm resistance vessels. The attenuation of BQ-123 mediated vasodilatation by BQ-788 could simply be an additive effect of BQ-788 mediated constriction on the BQ-123 mediated vasodilatation. However, in the current study, it reflects the combined effects of ETA and ET_B receptor blockade, indicating the potential for ETA receptor antagonists may offer more benefit in terms of vasodilatation than combined ETA/ET_B receptor antagonists.

The vasoconstrictor effects of ET_B receptor antagonism may result directly from blockade of the vasodilator effects of the endothelial ET_B receptor or indirectly from displacement of endogenously generated ET-1 from ET_B receptors to unoccupied ET_A receptors. Clearly, the indirect effects of ET-1 on ET_A receptors are more relevant with administration of selective ET_B antagonists than with non-selective ET_A/ET_B receptor antagonists, because in this latter situation the constrictor ET_A receptor is also blocked. Indeed, vasodilator effects have been demonstrated with both selective (Ferro, et al., 1996; Haynes and Webb, 1994) and non-selective (Ferro, et al., 1997; Haynes, et al., 1996) endothelin receptor antagonists in humans and the non-selective ET_A/ET_B antagonist bosentan has recently been shown to effectively lower blood pressure in patients with hypertension (Krum, et al., 1998). In the current study, the vasodilator effects of BQ-123 are attenuated in the presence of BQ-788, suggesting that the overall effect of vascular ETB receptor stimulation by endogenous ET-1 is vasodilatation. This

attenuation of BQ-123 mediated vasodilatation by BQ-788 suggests that the vasoconstrictor effect of ET_B receptor blockade is not mediated by displacement of ET-1 onto the ET_A receptor, but is due to direct blockade of ET_B mediated vasodilator tone.

Results from previous local studies, using a 'nitric oxide clamp technique'(Stroes, et al., 1995), suggest that the vasodilator response to BQ-123 is in part due to blockade of ET_A constrictor tone, and in part mediated by nitric oxide (Verhaar, et al., 1998) probably mediated by the endothelial ET_B receptor. Indeed in Chapter 5, the dilator effect of high dose infusion of the ET_B receptor agonist SFTX6c was shown to be mediated by the ET_B receptor through increased generation of nitric oxide, and not prostacyclin. These data highlight the importance of nitric oxide in ET_B receptor mediated dilatation. Loss of endothelial cell ET_B mediated vasodilator tone may occur in cardiovascular diseases, such as essential hypertension and hypercholesterolaemia, in which there is associated endothelial dysfunction (Casino, et al., 1995; Panza, et al., 1995). Here, because of a reduced capacity for ET_B receptor mediated, nitric oxide dependent dilatation, selective ET_A receptor antagonists may be less effective.

Local vasoconstriction in response to infusion of BQ-788 alone was demonstrated in the current study, suggesting that the balance of effects of ET-1 at the vascular ET_B receptors favours vasodilatation. This is further supported by the fact that co-infusion of the ET_B receptor antagonist, BQ-788, reduces the vasodilator response to BQ-123, and by the suggestion that the degree of vasodilatation to the combined ET_A/ET_B endothelin receptor antagonist, TAK-044 (Haynes, et al., 1996) appears to be less than that to the ET_A selective agent, BQ-123 (Haynes and Webb, 1994). It is possible that the predominant effects of intra-luminal infusion of BQ-788 selectively affects the endothelial ET_B receptor because the drug has better access to the endothelial than to the smooth muscle receptors. However, this is unlikely because ET-1 and BQ-123 find ready access to the smooth muscle. The response to BQ-788 may indicate either

displacement of ET-1 from, or failure of clearance of ET-1 by, ET_B receptors (Plumpton, et al., 1996). However, the present study cannot distinguish between these effects. Unfortunately, there are currently no pharmacological tools available that have been shown clearly to distinguish between the endothelial and vascular smooth muscle ET_B receptors.

Although the balance of effects of endogenous ET-1 at ET_B receptors would appear to be vasodilatation, partly mediated by nitric oxide, earlier studies describe venoconstriction and vasoconstriction in response to exogenous administration of selective ET_B receptor agonists (Chapter 3, Chapter 4 and Chapter 5; Strachan, et al., 2000; Haynes, et al., 1995; Strachan, et al., 1995). This constriction may be of functional importance, especially in cardiovascular disease. Interestingly, in ischaemic heart disease there appears to be upregulation of human coronary ET_B receptors (Dagassan, et al., 1996) and this is associated, in heart failure, with enhanced vasoconstrictor responses to sarafotoxin S6c in both the forearm (Love, et al., 1996) and coronary circulation (Cannan, et al., 1996). The endothelial ET_B receptor, however, appears still to be functionally active because infusion of BQ-788 has been shown to cause forearm vasoconstriction in patients with heart failure (Love, et al., 2000).

In diseases associated with endothelial dysfunction, such as hypercholesterolemia and hypertension (Casino, et al., 1995; Panza, et al., 1995), while selective ET_A receptor blockade should allow antagonism of direct ET_A receptor mediated constriction, the beneficial effects of ET_B mediated vasodilator tone may be reduced or absent and the constrictor effects of the vascular smooth muscle ET_B receptor more important. Therefore, the response to ET_A receptor blockade may be decreased in such conditions. In contrast, there is evidence to support upregulation of non-constrictor ET_B receptors in renal disease [see (Rabelink, et al., 1996) for discussion] which again has important

implications in the use of endothelin antagonists as treatments in cardiovascular disease. Clearly, at some stage, it will be necessary to examine the integrated physiology of systemic ET_A and ET_B blockade in order to fully understand the relative importance of these receptor subtypes in physiological control of the circulation.

Chapter 7

The repeatability of local forearm vasoconstriction to endothelin-1 measured by venous occlusion plethysmography

Repeatability of local forearm vasoconstriction to endothelin-1 measured by venous occlusion plethysmography. Strachan FE, Newby DE, Sciberras DG, M^{C}Crea JB, Goldberg MR, Webb DJ. SUBMITTED FOR PUBLICATION

7.1 Introduction

The importance of ET-1 as an endogenous mediator of vascular tone has been confirmed by forearm (Haynes, et al., 1996; Haynes and Webb, 1994) and systemic (Haynes, et al., 1996; Spratt, et al., 1999) vasodilatation in response to local and systemic administration of endothelin receptor antagonists, respectively. As a consequence of its potent vasoconstrictor and growth promoting properties, ET-1 has been implicated in the pathophysiology of diseases such as hypertension, heart failure and renal failure (Battistini, et al., 1993; Haynes and Webb, 1993), leading to the rapid development of endothelin receptor antagonists as potential vasodilator treatments for cardiovascular disease (Strachan and Webb, 1998). Some of these compounds are currently being investigated in clinical trials (Freed, et al., 1999; Haynes, et al., 1996; Weber, et al., 1996).

Venous occlusion plethysmography coupled with brachial artery infusion provides a valuable method for the assessment of pharmacological and physiological vasoactive properties of locally active doses of potentially vasoactive compounds (Webb, 1995). Indeed, vasoconstriction of the forearm vascular bed to local intra-arterial infusion of ET-1 has previously been demonstrated by venous occlusion plethysmography (Clarke, et al., 1989; Haynes, et al., 1995; Haynes and Webb, 1994) and inhibition of this response has been used to assess the efficacy of endothelin receptor antagonists in early clinical trials (Ferro, et al., 1997; Haynes, et al., 1996). However, although there are published data describing the repeatability of this technique with other vasoactive agents or stimuli in forearm resistance vessels *in vivo* (Newby, et al., 1997; Petrie, et al., 1998; Roberts, et al., 1986; Walker, et al., 1999), there are currently no data specifically with ET-1.

In order to assess the repeatability of the forearm blood flow response to intra-arterial infusion of ET-1 *in vivo*, we compared the response to infusion of two locally active doses of ET-1, administered on separate occasions, in the forearm resistance vessels of healthy men. Each dose level was administered twice, on consecutive visits, and the dose order was randomised. We also compared methods of data presentation of the forearm blood flow data to assess which method is more reliable in this setting.

7.2 Methods

7.2.1 Subjects

Eight healthy men (aged between 18-60 years), were recruited to the study, under the standard conditions described in Chapter 2.

7.2.2 Drugs

Endothelin-1 was administered by continuous infusion, via the brachial artery, for 120 min at a rate of 2.5 or 10 pmol/min according to the study randomisation. The infusion rate was kept constant at 1 ml/min for both dose levels. We have previously assessed the effects of ET-1 at a non-systemic dose of 5 pmol/min for 60 - 90 min (Ferro, et al., 1997; Haynes, et al., 1996; Haynes and Webb, 1994). The doses selected for the current study allowed assessment of the repeatability of the response to ET-1 at a lower (2.5 pmol/min) and a higher (10 pmol/min) dose than previously used, to provide further information on the threshold of effect, the dose response and tolerability.

All dilutions were prepared in 0.9% saline (Baxter Healthcare Ltd, Thetford, UK) from sterile stock solutions on the day of the study.

Intra-arterial infusion

The brachial artery of the non-dominant arm was cannulated under local anaesthetic (1% lignocaine; Astra Pharmaceuticals, Kings Langley, England) with a 27 SWG steel

cannula (Cooper's Needle Works, Birmingham, UK). The infusion rate was kept constant at 1 ml/min throughout.

7.2.3 Measurements

Forearm Blood Flow

The response to intra-arterial infusion was assessed by measurement of forearm blood flow in both the infused and non-infused forearms by venous occlusion plethysmography as described in Chapter 2.

Blood Pressure and Heart Rate

Blood pressure and heart rate were measured in the non-infused arm using a well-validated semi-automated non-invasive method (Wiinberg, et al., 1988). Blood pressure was measured immediately after forearm blood flow to avoid any effect on these measurements of the venous congestion caused by this procedure.

7.2.4 Study Design

In a single-blind, randomised, 2 way crossover study, the local effects of two dose levels of ET-1 were investigated in eight healthy men. On separate occasions, each separated by at least one week, subjects received an intra-arterial infusion of ET-1 (2.5 pmol/min or 10 pmol/min) for 120 min. Each dose level was administered twice, on consecutive visits and the dose order randomised. Subjects were blinded to the dose administered.

Saline (0.9%) was infused for at least 30 min, during which 3 measurements of forearm blood flow were made. ET-1 was then infused for 120 min. Forearm blood flow, and blood pressure and heart rate recordings, were made at 10 min intervals throughout.

7.2.5 Statistical Analysis

Plethysmographic data listings were extracted from data files and forearm blood flows calculated as described in Chapter 2. Baseline blood flow was taken as the last measurement during the saline infusion, before the start of the ET-1 infusion. Forearm blood flow results are expressed as the % change from baseline in the ratio of blood flow between the infused and non-infused arms (Webb, 1995). We also expressed the results as % change in forearm blood flow in the infused arm alone, to compare this with our standard method of data presentation. The area under the curve (AUC) was calculated for both methods to present a summary statistic for the overall response.

The repeatability of each method was assessed by the method of Bland and Altman (Bland and Altman, 1986) using Student's *t* distribution. In brief, the mean response and the mean difference between the responses on each visit are compared and the repeatability coefficient calculated according to the recommendations of the British Standards Institution (Bland and Altman, 1986). Power calculations were performed using the standard deviation and the mean response, as a % change, for visit 1 for each dose, to estimate the sample sizes required to detect a shift in the response at 60, 90 and 120 min; and the AUC for (0 - 60 min), (30 - 60 min), (0 - 90 min), (60 - 90 min), (0 - 120 min) and (90 - 120); for each dose for 80 or 90% power to detect a predetermined difference (of 10 - 100%) with significance accepted at the 5% level.

All results are expressed as mean ± standard error of the mean (SEM). Blood pressure, heart rate and baseline measurements were compared using the Student's paired *t*-test. Forearm blood flow data were examined by repeated-measures analysis of variance (ANOVA) (Excel 5.0, Microsoft Ltd, Wokingham, UK). Statistical significance was accepted at the 5% level.

7.3 Results

All eight subjects successfully completed the study (age range: 18-50 years, mean 33 ± 3 years). There were no significant differences between baseline measurements on each of the study visits. There was no significant change in blood pressure or heart rate at the end of each infusion (Table 1). There was a trend for the blood flow in the non-infused arm to increase with time (Figure 1). Although this trend reached significance over 120 min ($p=0.02$) for one of the visits (10 pmol/min, visit 1) when the data were expressed as % change in blood flow, there was no significant change in forearm blood flow (absolute values) in the non-infused arm at the end of each infusion (Table 1).

Although there were no reported adverse events with either dose of ET-1, some skin blanching was noted in some volunteers with the higher dose of ET-1 (10 pmol/min). These effects were not formally evaluated and did not cause symptoms at the end of the study or thereafter.

Table 7.1 Mean arterial pressure (MAP), heart rate (HR) and forearm blood flow (FBF) at baseline and 120 min after the start of each infusion. Values are mean ± SEM.

	Endothelin-1 infusion			
	2.5 pmol/min		**10 pmol/min**	
	visit 1	**visit 2**	**visit 1**	**visit 2**
MAP (mmHg)				
Basal	94 ± 2	92 ± 3	91 ± 3	90 ± 2
120 Mins	94 ± 3	98 ± 4	93 ± 4	94 ± 3
HR (bpm)				
Basal	62 ± 4	64 ± 5	61 ± 5	63 ± 4
120 Mins	58 ± 4	64 ± 7	62 ± 4	63 ± 4
FBF (ml/100ml/min)				
Control Arm				
Basal	3.4 ± 0.3	3.3 ± 0.4	3.1 ± 0.3	3.5 ± 0.3
120 min	3.7 ± 0.5	3.9 ± 0.3	4.1 ± 0.3	4.4 ± 0.5
Infused Arm				
Basal	4.0 ± 0.4	3.9 ± 0.7	3.6 ± 0.4	3.8 ± 0.5
120 min	2.5 ± 0.3	3.2 ± 0.1	1.6 ± 0.1	1.7 ± 0.2

Forearm blood flow

(i) Forearm blood flow ratio (FBF_R)

Forearm vasoconstriction was indicated by a reduction in the ratio of forearm blood flow in response to both doses of ET-1 at each visit ($p<0.0001$, all visits) (Figure 7.1). The response to ET-1 was slow in onset and appeared to plateau at around 60 min. There was no significant difference between the responses on visit 1 and visit 2 for either dose. The repeatability coefficient for 10 pmol/min was lower than that for 2.5 pmol/min (Table 7.2), indicating that the response to 10 pmol/min was the more repeatable.

The AUC was calculated for each dose and each visit ($p<0.0001$, all visits) (Table 7.2). There was no significant difference between the responses on visit 1 and visit 2 for either dose. Again, the repeatability coefficient for 10 pmol/min was lower than for 2.5 pmol/min (Table 7.2).

(ii) Infused arm only (FBF_I)

There was a significant reduction in blood flow in the infused arm in response to both doses of ET-1 at each visit ($p<0.0001$, all visits) (Figure 7.1). Although there was no significant difference between the response to ET-1 (10 pmol/min) on visit 1 and visit 2 ($p=0.7$), the difference between the response to ET-1 (2.5 pmol/min) on visit 1 and visit 2 was significant ($p=0.03$). The repeatability coefficient for 10 pmol/min was lower than that for 2.5 pmol/min (Table 7.2).

The AUC was calculated for each dose and each visit ($p<0.0001$, all visits) (Figure 7.1). There was no significant difference between the responses on visit 1 and visit 2 for either dose. Again, the repeatability coefficient for 10 pmol/min was lower than that for 2.5 pmol/min (Table 7.2).

(iii) Non-infused arm only(FBF_{NI})

Although there was no significant change in forearm blood flow (absolute values) at the end of the infusion (Table 7.1), when expressed as a % change from baseline, the change on visit 1 for the 10 pmol/min dose was significant over 120 min ($p=0.02$; ANOVA, one way). This change was not significant up to and including the 90 min timepoint for this visit ($p>0.1$; ANOVA, one way), or for the other visits over 120 min.

There was no significant difference between the % change in the non-infused arm on visit 1 and visit 2 for the 10 pmol/min dose. However the difference between the % change on visit 1 and visit 2 for the 2.5 pmol/min dose reached statistical significance ($p=0.047$).

(iv) Power calculations

The sample sizes estimated from the power calculations were smaller when data were represented as a % change in the ratio of forearm blood flow rather than as % change in the infused arm alone (Tables 7.3 & 7.4).

Figure 7.1 Response of forearm blood flow to local intra-arterial infusion of ET-1; % change in forearm blood flow ratio; infused arm:non-infused arm (2.5 pmol/min; visit 1, open circles; visit 2, closed circles and 10pmol/min; visit 1, open squares; visit 2, closed squares). Responses are expressed as mean % change in forearm blood flow ratio ± SEM.

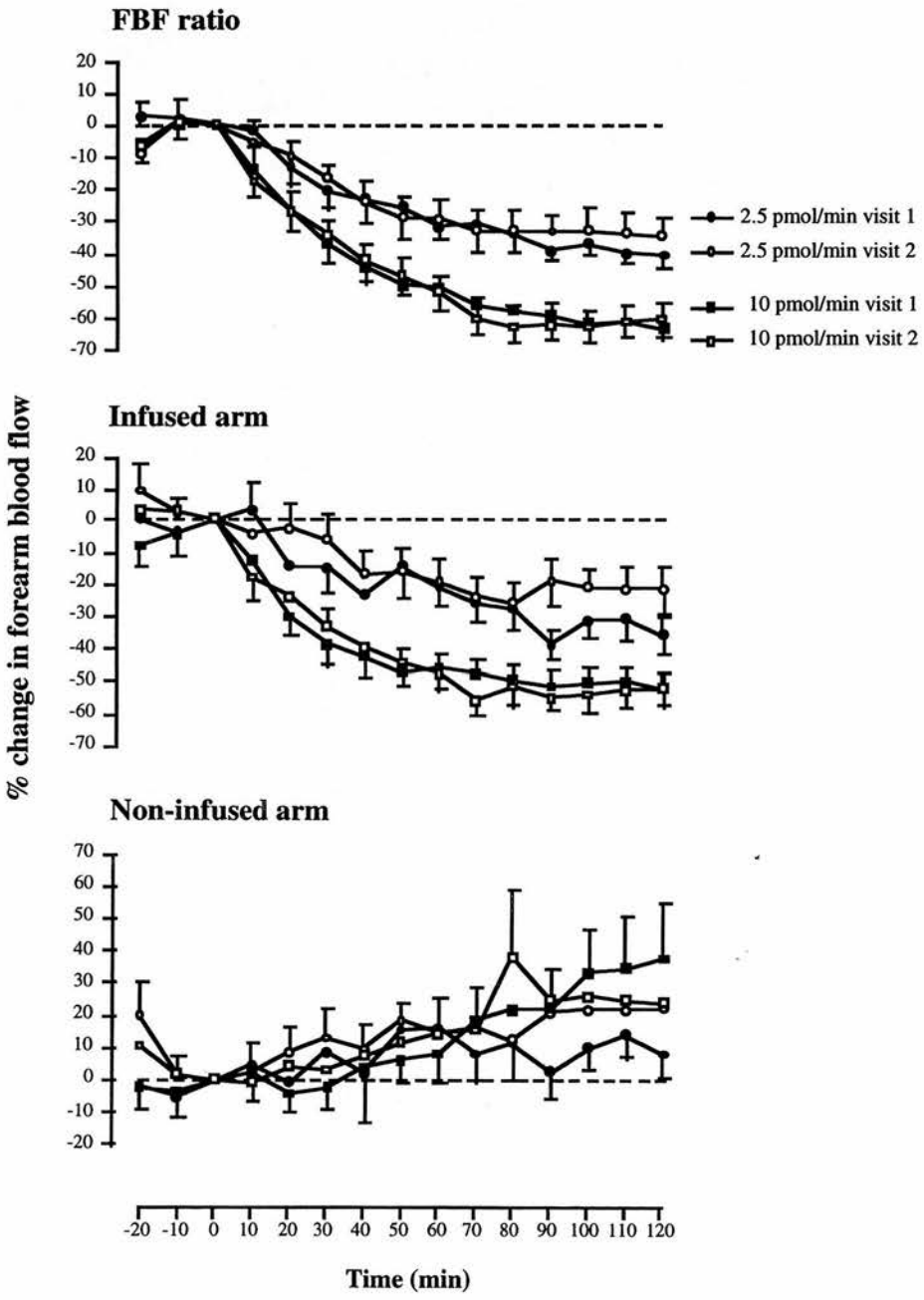


Table 7.2 Data for % change in forearm blood flow (FBF) ratio, blood flow in the infused arm for 30, 60, 90 and 120 min following the start of each infusion with AUC, confidence intervals and repeatability coefficients.

		FBF ratio (infused:non-infused arm)			FBF infused arm		
ET-1 (pmol/min)	**Time** (min)	**Mean % change** (visit 1 & visit 2)	**Mean difference** (visit 1 - visit 2)	**Repeatability coefficient** (%)	**Mean % change** (visit 1 & visit 2)	**Mean difference** (visit 1 - visit 2)	**Repeatability coefficient** (%)
2.5	30	-19	-4	15	-11	-9	20
	60	-30	-2	14	-20	-2	19
	90	-36	-6	14	-28	-20	19
	120	-38	-6	15	-29	-14	21
	AUC	-3064	-201	1411	-2222	-697	1774
10	30	-36	-3	16	-36	-6	15
	60	-51	2	10	-47	1	12
	90	-61	3	8	-53	4	12
	120	-62	-3	11	-52	1	13
	AUC	-5534	96	1003	-4957	83	1259

Table 7.3 Power calculations estimating the sample sizes required to detect a 10, 25, 33, 50, 75 and 100% shift in the response, as % change (i) at 120 min, (ii) AUC (0 - 120 min), (iii) AUC (90 - 120) min; for each dose with a power of 90% and 80% and significance accepted at 5%.

(i)

		FBF - ratio		**FBF - Infused arm**	
ET-1 (pmol/min)	**% shift** (120 min)	**90% power**	**80% power**	**90% power**	**80% power**
2.5	10	132	99	389	293
	25	22	16	63	47
	33	13	10	36	27
	50	6	≥ 5	16	12
	75	≥ 5	≥ 5	7	6
	100	≥ 5	≥ 5	≥ 5	≥ 5
10	10	38	29	122	92
	25	6	≥ 5	20	15
	33	≥ 5	≥ 5	12	9
	50	≥ 5	≥ 5	≥ 5	≥ 5
	75	≥ 5	≥ 5	≥ 5	≥ 5
	100	≥ 5	≥ 5	≥ 5	≥ 5

(ii)

		FBF - ratio		FBF - Infused arm	
ET-1 (pmol/min)	**% shift** (AUC 0-120 min)	**90% power**	**80% power**	**90% power**	**80% power**
2.5	10	180	136	1093	823
	25	29	22	175	132
	33	17	13	101	76
	50	8	6	44	33
	75	≥ 5	≥ 5	20	15
	100	≥ 5	≥ 5	11	9
10	10	33	25	144	108
	25	6	≥ 5	23	18
	33	≥ 5	≥ 5	14	10
	50	≥ 5	≥ 5	6	≥ 5
	75	≥ 5	≥ 5	≥ 5	≥ 5
	100	≥ 5	≥ 5	≥ 5	≥ 5

(iii)

		FBF - ratio		FBF - Infused arm	
ET-1 (pmol/min)	**% shift** (AUC 90-120 min)	**90% power**	**80% power**	**90% power**	**80% power**
2.5	10	83	63	368	277
	25	13	10	59	45
	33	8	6	34	26
	50	≥ 5	≥ 5	15	12
	75	≥ 5	≥ 5	7	≥ 5
	100	≥ 5	≥ 5	≥ 5	≥ 5
10	10	50	38	130	98
	25	8	6	21	16
	33	≥ 5	≥ 5	12	9
	50	≥ 5	≥ 5	6	≥ 5
	75	≥ 5	≥ 5	≥ 5	≥ 5
	100	≥ 5	≥ 5	≥ 5	≥ 5

Table 7.4 Power calculations estimating the sample sizes required to detect a 25 and 50% shift in the response, as % change at 60 min, 90 min and 120 min and % change in AUC (0 - 60 min), (30 - 60 min), (0 - 90 min), (60 - 90 min), (0 - 120 min) and (90 - 120), for each dose with a power of 90% and significance accepted at 5%.

ET-1 (pmol/min)	FBF method	% shift	AUC 0 - 60 min	AUC 30 - 60 min	60 min	AUC 0 - 90 min	AUC 60 - 90 min	90 min	AUC 0 - 120 min	AUC 90 - 120 min	120 min
2.5	**ratio**	25	116	59	30	52	22	20	29	13	22
		50	29	15	8	13	6	≥5	8	≥5	6
	infused	25	726	369	165	296	92	165	175	59	6
	arm	50	182	93	42	74	23	42	44	15	≥5
10	**ratio**	25	42	15	≥5	13	8	12	6	8	6
		50	11	≥5	≥5	≥5	≥5	≥5	≥5	≥5	≥5
	infused	25	59	31	27	34	22	27	23	21	15
	arm	50	15	8	7	9	6	7	6	6	≥5

7.4 Discussion

These results describe dose-dependent vasoconstriction in the forearm vascular bed of healthy men in response to infusion of locally active doses of ET-1 (2.5 and 10 pmol/min), consistent in evolution and magnitude with previous responses to ET-1 (1 and 5 pmol/min) (Clarke, et al., 1989; Haynes, et al., 1996; Haynes, et al., 1995). The response to each dose of ET-1 was investigated on two separate occasions and found to be similar on both visits.

Forearm blood flow can be measured in both arms and it has been suggested that data from studies with locally active doses of drugs are best represented as % change in the ratio of forearm blood flow in the infused and non-infused arm (Benjamin, et al., 1995; Chin-Dusting, et al., 1999; Petrie, et al., 1998; Webb, 1995). However, some investigators prefer to report the effects in the infused arm without including blood flow data from the non-infused arm (Panza, et al., 1990; Walker, et al., 1999). It is likely that, where the responses are relatively small, and particularily in the case of constrictors, the response is more accurately expressed as a % change in the ratio of forearm blood flow. On the other hand, when responses are potentially of a greater magnitude, particularily with vasodilator effects (Walker, et al., 1999), it may be more useful to express the response as a % change in the infused arm and use the effects in the non-infused arm only to confirm that there are no systemic drug effects.

We examined the response to ET-1 when expressed as % change in the ratio of forearm blood flow and as % change in the infused arm alone, to identify which is better suited for future studies investigating this response. Although both methods demonstrated significant reduction in blood flow for all visits (Figure 1), the repeatability coefficients were consistently lower for the blood flow ratio than for blood flow in the infused arm (Table 2) indicating that the data are less variable when presented as % change in forearm blood flow ratio. This translates into a need for a

lower sample size to achieve the same power, using blood flow ratios rather than changes in the infused arm alone (Table 3), suggesting that, at least in the case of responses to ET-1, presentation of the response as a ratio is more robust.

Summarising the forearm blood flow responses as areas under the curve (AUCs) allows presentation of responses as single values and may reduce the effect of single unexpected variations in blood flow on an overall assessment of the response. Again, the AUC data for the blood flow ratio appeared more robust than the AUC for the infused arm only with the response more repeatable and with consistently smaller sample sizes needed for future studies investigating this response. Interestingly, the sample sizes were smaller for AUC for the last 30 min of the response than for the AUC for the entire infusion period, for both the ratio and infused arm data (Table 4), probably because the response is more completely developed by this time.

In the current study there was a trend for the blood flow to increase in the non-infused arm, with significant change detected over 120 min on one of the study visits (10 pmol/min, visit 1). It is important to note that, although changes were observed in the non-infused arm, these changes were not significant up to 90 min. Therefore, limiting the duration of the intra-arterial infusion to 90 min or less might help avoid this problem. This increase in blood flow may simply be a time-dependent effect. However, as these effects were more pronounced with the higher dose level of ET-1 (10 pmol/min), it is possible that the changes described in the non-infused arm are a breakthrough of systemic effects. Previous investigations of forearm blood flow responses to ET-1 have demonstrated vasodilatation in response to low dose (~0.05 - 0.2 pmol/min) infusion of ET-1 (Dahlof, et al., 1990; Kiowski, et al., 1991). Therefore the small increase in blood flow observed in the non-infused arm during infusion of ET-1 (10 pmol/min) in the current study may have resulted from a small increase in ET-1 concentrations in the non-infused arm. Alternatively, this increase in

forearm blood flow could reflect splanchnic vasoconstriction with the higher dose of ET-1. Although no changes were seen in blood pressure in the non-infused arm during infusion of ET-1, the increase in blood flow in the non-infused arm could result from an increase in blood flow to the forearm vascular bed in response to a degree of systemic vasoconstriction in more important vascular beds. Indeed, similar effects have been described in studies with systemic infusion of angiotensin II, where forearm vasodilatation was demonstrated in response to high dose infusion of angiotensin II (Motwani and Struthers, 1992).

We have shown that the forearm blood flow response to intra-arterial infusion of ET-1, at two dose levels, is repeatable in between-day comparisons in healthy volunteers. These data support the use of the forearm blood flow response to ET-1 as a model to assess the effects of antagonists and inhibitors of the endothelin system in early clinical trials (Ferro, et al., 1997; Haynes, et al., 1996; Haynes and Webb, 1994). From our experience this model is most powerful when the response to intra-arterial infusion is assessed in a within-subject, placebo-controlled design, allowing the shift in individual dose responses to be assessed with each study subject acting as his own control. The method described is generally well tolerated and repeat studies can be scheduled 5-7 days apart, allowing the effects of study drug to be investigated using a crossover design. This model is also useful in assessing any differences in responses to ET-1 between patients and healthy matched controls (Hand, et al., 1999; Love, et al., 1996; Newby, et al., 1998).

The characteristically slow onset of and sustained vasoconstriction to ET-1 (Clarke, et al., 1989; Haynes, et al., 1995) precludes the construction of a full dose response curve on a single visit. There is also the possibility of an accumulation of effect with subsequent increases in dose level. Indeed, early studies with ET-1 demonstrated significant adverse events including vomiting, sensation of heat and deep muscular

pain following intra-arterial infusion of ET-1 in stepwise increases in dose from 5 x 10^{-11} to 5 x 10^{-8} mol/min with effects still evident 10 hours following infusion (Dahlof, et al., 1990). In the current study, we observed skin blanching in the infused arm following infusion of ET-1, but only at the higher dose level. These effects have not been noted in our studies with ET-1 (5 pmol/min) (Ferro, et al., 1997; Hand, et al., 1999; Haynes, et al., 1996; Haynes and Webb, 1994; Love, et al., 1996). In addition, a small but significant increase in forearm blood flow was noted in the non-infused arm following infusion of ET-1 (10 pmol/min), which could indicate threshold systemic effects at this dose level. Given these observations with ET-1 (10 pmol/min), and our previous results with ET-1 (5 pmol/min), we would suggest that the forearm blood flow response to intra-arterial infusion of ET-1 at 5 pmol/min for 90 min provides the most useful model for the assessment of effects of receptor antagonists or the responsiveness to ET-1 in patients. Based on power calculations from the current data, a sample size of 8 should be sufficient to detect a 50% shift in AUC (60 - 90 min) for ET-1 (10 pmol/min) and a 33% shift in AUC (60 - 90 min) for ET-1 (2.5 pmol/min), with 90% power, when responses are expressed as a % change in the forearm blood flow ratio. Therefore, we would expect that a sample size of 8 should be sufficient to detect a 33 - 50% shift in AUC (60 - 90 min) for 5 pmol/min, with 90% power, when responses are expressed as a % change in the forearm blood flow ratio.

In summary, the assessment of the forearm blood flow response to intra-arterial infusion of ET-1 provides a well tolerated and reliable model for the pharmacodynamic assessment of endothelin receptor antagonists at an early stage in drug development in a relatively small number of patients or controls (Ferro, et al., 1997; Hand, et al., 1999; Haynes, et al., 1996; Love, et al., 1996; Newby, et al., 1998) enabling identification of a pharmacologically effective dose range for use in early patient trials. The data from the current study should provide a valuable basis for protocol design in

early clinical pharmacology studies investigating the activity of new endothelin receptor antagonists.

Chapter 8

The effect of the non-selective endothelin receptor antagonist L-753,037 on forearm vasoconstriction to endothelin-1

8.1 Introduction

As discussed previously, the importance of endothelin-1 (ET-1) as a physiological mediator of basal vascular tone *in vivo* in man has been demonstrated by local (Haynes, et al., 1996; Haynes, et al., 1994; Verhaar, et al., 1998) and systemic (Freed, et al., 1999; Haynes, et al., 1996; Spratt, et al., 1999; Weber, et al., 1996) vasodilatation in response to endothelin receptor antagonism in healthy subjects. The potent vasoconstrictor effects of ET-1 (Clarke, et al., 1989; Yanagisawa, et al., 1988), combined with the increased plasma concentrations of ET-1 in patients with cardiovascular disease, including cardiac (Pacher, et al., 1993) and renal failure (Koyama, et al., 1989), provide strong evidence to support a functional role for ET-1 in the development and maintenance of the increased peripheral vascular resistance associated with these conditions. Indeed, vasodilator effects of endothelin antagonists have been described in patients with heart failure (Cowburn, et al., 1998; Kiowski, et al., 1995; Love, et al., 1996) and hypertension (Cardillo, et al., 1999; Ferro, et al., 1996; Taddei, et al., 1999).

The recognition of the endothelin system as a therapeutic target in the treatment of cardiovascular disease has led to the rapid development of endothelin receptor antagonists as potential vasodilator treatments (Strachan and Webb, 1998), with a number of these compounds, both ET_A receptor selective and mixed ET_A/ET_B receptor antagonists, currently being investigated in clinical trials. L-753,037 is a potent, non-peptide, mixed ET_A/ET_B receptor antagonist (Zhao, et al., 1999) which, unusually, is almost equipotent at both receptors.

Following validation of the forearm blood flow response to ET-1 as a model for the assessment of endothelin receptor antagonists in early clinical trials (Chapter 7), the effect of L-753,037 on forearm vasoconstriction to ET-1 in healthy volunteers was

investigated. In addition the effect of L-753,037 on blood pressure, heart rate and plasma concentration of ET-1 was assessed.

8.2 Methods

8.2.1 Subjects

Eight healthy men, aged between 18 and 60 years, were recruited to the study which was performed in the Clinical Research Centre at the Western General Hospital, Edinburgh under the standard conditions listed in Chapter 2. All subjects attended for a pre-trial screening visit for full assessment of medical history and a physical examination. All subjects tested negative for HBsAg and for drugs of abuse. No subject received any medication, including over the counter medications, in the 2 weeks before each treatment period. All subjects abstained from alcohol for 48 hours and from food, caffeine containing drinks and tobacco for at least 7 hours before L-753,037 and ET-1 administration. All studies were performed in a quiet room kept at a controlled temperature between 22-24°C.

8.2.2 Drugs

Intra-arterial administration of ET-1

Endothelin-1 (Calbiochem, Nottingham, UK) was administered by continuous intra-arterial infusion for 90 min at an infusion rate of 5 pmol/min, as described previously (Ferro, et al., 1997; Haynes, et al., 1996, and Chapter 7; Haynes and Webb, 1994), ET-1 administration was unblinded. Saline (0.9%) was infused for at least 30 min before ET-1 infusion.

Intravenous infusion of L-753,037

On separate occasions, L-753,037 (0.25 or 0.375 mg/kg) or matching placebo was administered by IV infusion for 30 min.

Endothelin-1 was prepared in 0.9% saline (Baxter Healthcare Ltd) from sterile stock solutions on the day of the study. L-753,037 was prepared under standard aseptic conditions within the hospital pharmacy department on the evening before administration.

8.2.3 Measurements

Forearm blood flow

The response to brachial artery infusion was assessed in both forearms by measurement of blood flow using venous occlusion plethysmography, as described in Chapter 2. Recordings of forearm blood flow were made over 3 min periods at 10 min intervals throughout the intra-arterial infusion.

Blood pressure and heart rate

Blood pressure and heart rate were measured in the non-infused arm as described in Chapter 2. During intra-arterial infusions, blood pressure was measured immediately after forearm blood flow measurements to avoid any effect of the venous congestion caused by this procedure on these measurements.

Plasma ET-1

Blood samples for assay of ET-1 were obtained, via an 18 SWG cannula sited in the opposite arm from the intra-arterial infusion. In brief, 10 ml samples were collected into sterile EDTA tubes (K3 EDTA, Vacutainer, Becton Dickinson Vacutainer Systems, Europe) centrifuged immediately at 1000 g for 20 min (ET-1 assay) and stored in plain tubes at -80°C prior to assay. Endothelin-1 concentrations were determined by standard radioimmunoassay (Peninsula Laboratories Europe), as described previously (Hand, et al., 1999).

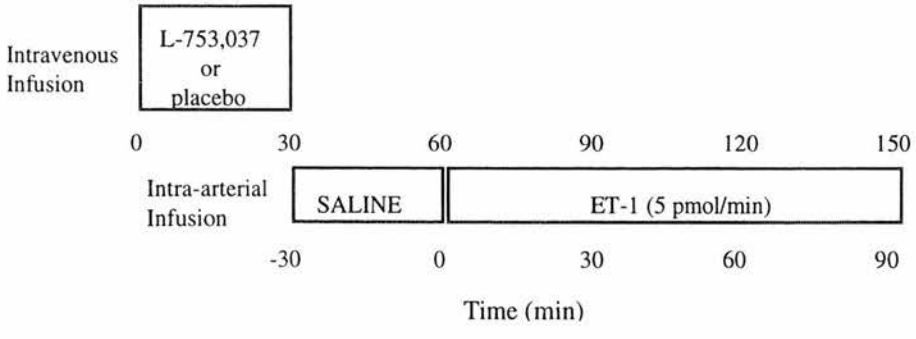
Additional blood samples were taken at screening, on admission, prior to discharge and at the post trial physical examination for standard biochemistry and haematology assays. Routine urinalysis was carried out at screening and after the trial .

8.2.4 Study design

In a 3 way, placebo controlled, crossover study, the effects of L-753,037 (0.25 or 0.375 mg/kg) or placebo on the local vasoconstrictor response to intra-arterial infusion of ET-1 and systemic haemodynamics were investigated, in 8 healthy male subjects. On separate occasions, each separated by at least one week, L-753,037 or matching placebo was administered by IV infusion for 30 min.

Blood pressure and heart rate were measured on 3 occasions in the 30 min before and at 30 min intervals until 60 min after the start of the infusion of L-753,037, thereafter recordings were made at 10 min intervals until the end of the intra-arterial infusion (150 min after the start of L-753,037 infusion) (Figure 8.1). Blood samples for assessment of plasma concentrations of ET-1 were collected immediately before and at 30 min intervals until 180 min after the start of the L-753,037 infusion.

Figure 8.1 Schematic representation of the study design: L-753,037 or placebo was infused intravenously for 30 min, ET-1 was infused intra-arterially for 90 min starting from 60 min after the start of the L-753,037 infusion.



Subjects attended the research centre on the morning of each study day and were discharged the following day, 24 hours after L-753,037 infusion. Blood pressure and heart rate were measured and a 12 lead ECG, physical examination, safety blood sample and adverse event check were performed before discharge.

8.2.5 Analysis

Forearm blood flow

Plethysmographic data listings were extracted from data files and forearm blood flows calculated for individual venous occlusion cuff inflations using a template spreadsheet (Excel 5.0; Microsoft Ltd, Wokingham, UK), as described in Chapter 2. Forearm blood flow results are expressed as the percentage change from baseline in the ratio of blood flow between the infused and non-infused arms (Webb, 1995; Benjamin, 1995; and Chapter 7). Data were examined by repeated-measures analysis of variance (ANOVA).

Blood pressure and heart rate

Blood pressure is presented as mean arterial pressure (MAP; diastolic blood pressure + 1/3 pulse pressure). Blood pressure and heart rate are expressed as placebo corrected percentage changes from baseline at 150 min after the start of the L-753,037 infusion (Haynes, et al., 1996). Statistical analysis was performed on untransformed data and data examined by repeated-measures analysis of variance (ANOVA).

Plasma ET-1

Plasma concentrations of ET-1 (pg/ml) are presented as absolute values at 150 min after the start of the L-753,037 infusion with data examined by repeated-measures analysis of variance (ANOVA).

All results are expressed as mean ± standard error of the mean (SEM). Data analysis was performed using an Excel data analysis package (Excel 5.0; Microsoft Ltd, Wokingham, UK). For ANOVA comparisons, treatment and subject were the factors and the p values are for treatment comparisons over all timepoints. Statistical significance was taken at the 5% level.

8.3 Results

Eight subjects (aged 20-42 years) completed the study. There were no serious or unexpected adverse events and no significant changes in safety evaluations. The most commonly reported event, on active treatment, was headache and abdominal rash. Other events reported included a fainting episode on placebo treatment and four events directly related to the intra-arterial infusion, which included blanching of skin and local swelling at the cannula site.

There was no significant change in blood pressure and heart rate, or in blood flow measured in the non-infused arm during the intra-arterial infusions, confirming that any effects of the intra-arterial infusion were confined to the infused arm (Table 8.1).

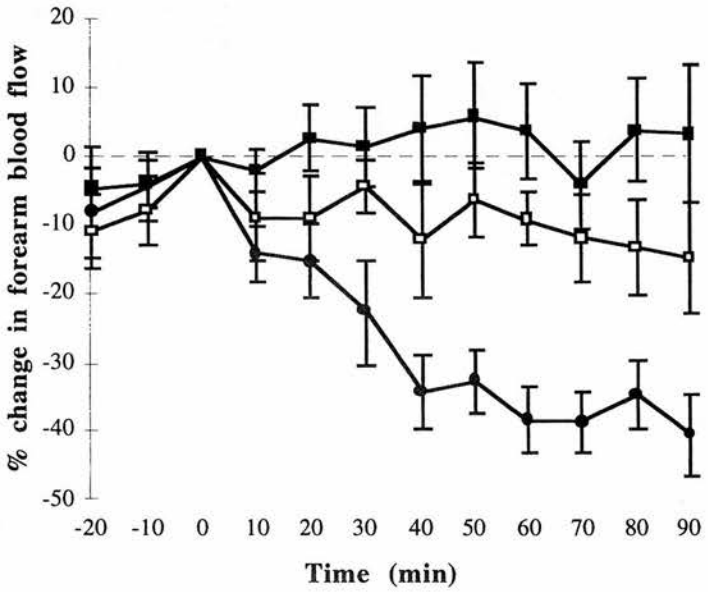
Forearm blood flow

There was significant local vasoconstriction, indicated by a reduction in the ratio of forearm blood flow, in response to intra-arterial infusion of ET-1 following placebo infusion (-40±6%, $p<0.0001$; Figure 8.2). Forearm vasoconstriction was significantly attenuated (-14±8%, $p<0.0001$ vs placebo) following infusion of L-753,037 (0.25 mg/kg) and completely abolished (3±10%, $p<0.0001$ vs placebo) following infusion of L-753,037 (0.375 mg/kg). The attenuation of the response to ET-1 was significantly greater with L-753,037 at 0.375 mg/kg than at 0.25 mg/kg ($p=0.0004$).

Table 8.1 Mean arterial pressure (MAP), heart rate (HR), plasma ET-1 and forearm blood flow (FBF) predose 60 min and 150 min after the start of the L-753,037 infusion. Values are mean ± SEM.

	Placebo	**L-753,037 (0.25 mg/kg)**	**L-753,037 (0.375 mg/kg)**
MAP (mmHg)			
Predose	88±2	93±2	86±3
60 min	90±1	89±3	84±3
150 min	93±2	93±4	83±3
HR (bpm)			
Predose	62±3	58±2	62±2
60 min	61±3	62±2	65±1
150 min	60±2	65±3	63±2
Plasma ET-1 (pg/ml)			
Predose	2.2±0.3	2.4±0.2	2.1±0.2
60 min	2.5±0.3	18.9±2.7	14.9±1.1
150 min	3.8±0.4	17.5±2.5	21.0±2.1
FBF (ml/100ml/min)			
Control Arm			
Predose	-	-	-
60 min	2.2±0.3	2.7±0.5	2.2±0.2
150 min	2.8±0.3	3.0±0.3	2.7±0.3
Infused Arm			
Predose	-	-	-
60 min	2.8±0.4	3.2±0.5	2.6±0.2
150 min	2.1±0.4	3.0±0.3	3.2±0.4

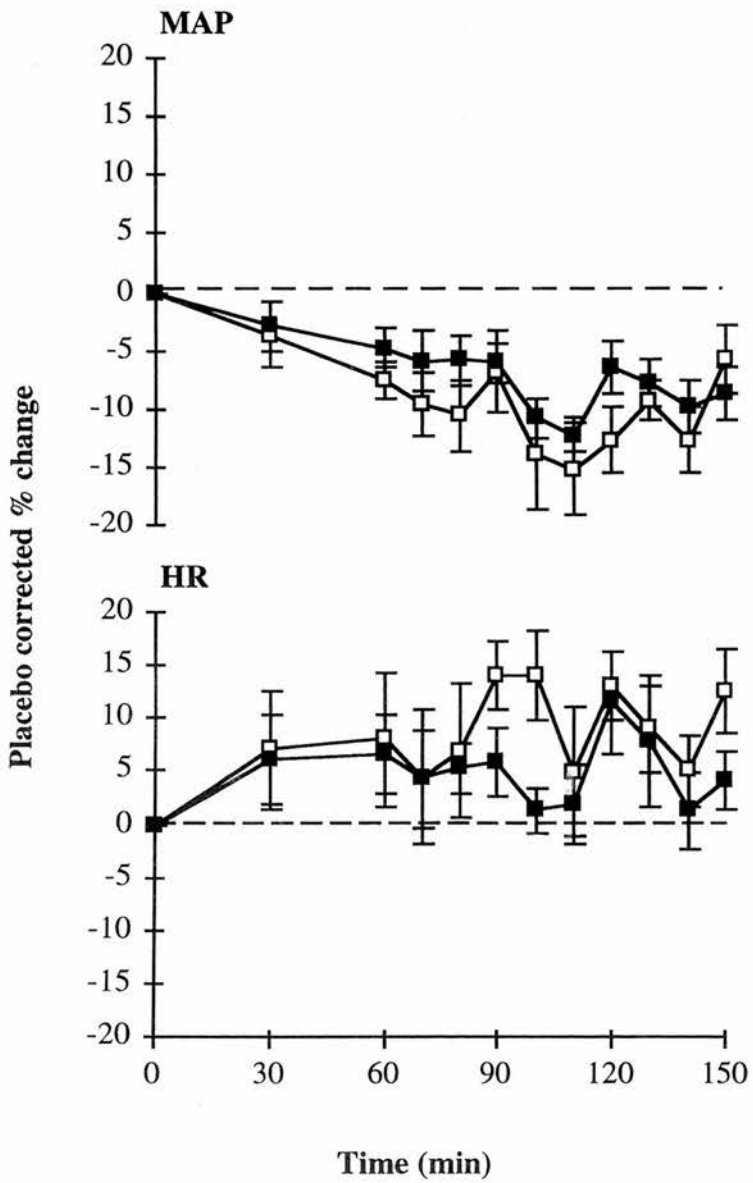
Figure 8.2 Response of forearm blood flow to local intra-arterial infusion of ET-1 (5 pmol/min) after iv administration of L-753,037 (0.25 mg/kg, open squares or 0.375 mg/kg, closed squares) or placebo, closed circles. Responses are expressed as mean % change in forearm blood flow ratio ± SEM.



Blood pressure and heart rate

There were no significant changes in blood pressure or heart rate after placebo administration (Table 8.1). However blood pressure was reduced following administration of L-753,037 (0.25 mg/kg: -6±3%; 0.375 mg/kg: -9±2%; $p<0.0001$ vs placebo) (Figure 8.3). There was no significant difference in the blood pressure response to either dose level. Heart rate was increased following administration of L-753,037 (0.25 mg/kg: 12±4%; 0.375 mg/kg: 4±3%; $p<0.0001$ vs placebo) (Figure 8.3). There was no significant difference between the blood pressure response to either dose of L-753,037.

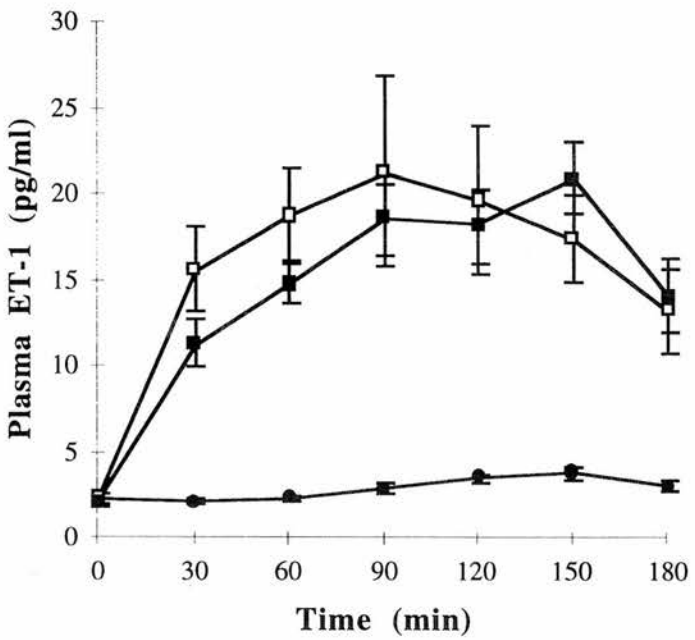
Figure 8.3 Mean arterial pressure and heart rate after iv administration of L-753,037 (0.25 mg/kg, open squares or 0.375 mg/kg, closed squares). Responses are expressed as placebo corrected mean % change ± SEM.



Plasma ET-1

Administration of placebo had no effect on plasma concentrations of ET-1 (Table 8.1), confirming that intra-arterial infusion of ET-1 had no effect on plasma concentrations of ET-1 measured in the opposite arm. In contrast, there was a significant increase in plasma concentrations of ET-1 150 min following administration of L-753,037 at both dose levels (0.25 mg/kg: 18±2 pg/ml; 0.375 mg/kg: 21±2 pg/ml; $p<0.0001$ for both) (Figure 8.4). There was no significant difference between the increase in plasma ET-1 to either dose of L-753,037.

Figure 8.4 Mean plasma concentrations of ET-1 (pg/ml) after iv administration of L-753,037 (0.25 mg/kg, open squares or 0.375 mg/kg, closed squares) or placebo, closed circles. Values are mean ± SEM.



8.4 Discussion

In the current study, vasoconstriction in the forearm vascular bed in response to intra-arterial infusion of a locally active dose of ET-1 (5 pmol/min) was demonstrated, consistent with previous results (Ferro, et al., 1997; Haynes, et al., 1996; Haynes and Webb, 1994; Strachan, et al., 1998). Systemic administration of the mixed ET_A/ET_B receptor antagonist L-753,037 significantly attenuated the response to ET-1 in the forearm vascular bed at two dose levels, completely blocking the response with the higher dose.

In addition to attenuating exogenous ET-1 mediated increases in forearm vascular tone, intravenous infusion of L-753,037 caused modest but significant reduction in blood pressure in healthy volunteers, indicating systemic vasodilatation. Unlike the attenuation of the forearm blood flow response, the reduction in blood pressure did not appear to be dose-dependent. There was also a significant increase in heart rate following infusion of L-753,037, most likely in response to systemic vasodilatation. Like the blood pressure effects, the change in heart rate did not appear to be dose dependent. The current design did not include measurement of cardiac index or stroke index, therefore it is not possible to calculate changes in peripheral vascular resistance in response to L-753,037 administration. However, the changes in blood pressure and heart rate are comparable to the effects seen previously with the non-selective ET receptor antagonist TAK-044 (Haynes, et al., 1996). The blood pressure changes demonstrated with TAK-044 were associated with a reduction in total peripheral vascular resistance. Therefore it is likely that blood pressure changes in the current study indicate systemic vasodilatation in response to L-753,037 administration.

Plasma concentrations of ET-1 have been shown to increase after administration of ET_B receptor selective antagonists (Cowburn, et al., 1998; Fukuroda, et al., 1994; Gratton, et al., 1997) and mixed ET_A/ET_B receptor antagonists (Haynes, et al., 1996;

Weber, et al., 1996) but not after adminstration of ET_A receptor selective antagonists (Ohnishi, et al., 1998; Spratt, et al., 1999), consistent with clearance of ET-1 mediated by the ET_B receptor. In the current study we found plasma ET-1 concentrations were increased after administration of L-753,037 at both dose levels, suggesting that the current dose range is active at the ET_B receptor. Interestingly, the increase in plasma concentrations of ET-1 did not appear to affect blood pressure or forearm blood flow responses.

The current dose range and method of administration appeared to be well tolerated as there were no serious or unexpected adverse events and few adverse events reported after administration of L-753,037. Headache is commonly reported with endothelin receptor antagonists, however, in the current study there was only one reported event of headache.

A number of ET_A receptor selective and mixed ET_A/ET_B receptor antagonists are currently being investigated in clinical trials (Strachan and Webb, 1998). It is unclear at present whether ET_A receptor selective or mixed ET_A/ET_B receptor antagonists will offer greater therapeutic benefit. In healthy volunteers (Verhaar, et al., 1998, Chapter 6) and from results in animal models (Gratton, et al., 1997), it would appear that the balance of effects at the vascular ET_B receptors favours vasodilatation. Therefore, selective ET_A receptor antagonists may provide greater vasodilatation in some circumstances by allowing blockade of the constrictor effects of the ET_A receptor while preserving the vasodilator effects of the ET_B receptor. Indeed, in heart failure patients, selective ET_B receptor antagonism results in both local (Love, et al., 1996) and systemic vasoconstriction (Cowburn, et al., 1998). Therefore, in heart failure selective ET_A receptor antagonists may offer greater therapeutic benefit. In contrast, in patients with hypertension, where endothelial function is likely to be impaired, vasodilatation has been described in response to selective ET_B receptor antagonism (Cardillo, et al.,

1999). This may reflect a reduced capacity for ET_B mediated vasodilatation through generation of nitric oxide (Taddei, et al., 1999). Therefore, in patient groups with endothelial dysfunction, mixed ET_A/ET_B receptor antagonists may be more effective vasodilator treatments. Further investigation in patient groups is required to assess the acute and chronic effects of selective ET_A receptor antagonists and mixed ET_A/ET_B receptor antagonists and to assess which of these approaches offers the greater therapeutic advantage in the clinical setting.

In summary, these results demonstrate that the combined ET_A/ET_B receptor antagonist L-753,037 is effective in blocking ET-1 mediated increase in vascular tone in healthy volunteers. In addition the reduction in blood pressure in comparison with placebo demonstrates the potential of L-753,037 as a vasodilator treatment. This study has allowed identification of a pharmacologically active, and apparently well tolerated, dose range for L-753,037 in healthy volunteers, this information may be of value in the design of future trials in patient groups.

Chapter 9

Vasodilator effects of the endothelin-A selective antagonist BMS-193884 in healthy men

9.1 Introduction

As discussed in Chapter 8, the recognition of the endothelin system as a new therapeutic target in the treatment of cardiovascular disease, has led to the rapid development of endothelin receptor antagonists as potential vasodilator treatments (Strachan and Webb, 1998), with a number of these compounds, both ET_A receptor selective and mixed ET_A/ET_B receptor antagonists, currently being investigated in clinical trials. Further investigation of the integrated cardiovascular effects in patient groups is required to address whether selective ET_A receptor antagonists would provide greater therapeutic benefit than mixed ET_A/ET_B receptor antagonists.

The forearm blood flow response to ET-1 provides a well validated model for the assessment of endothelin receptor antagonists in early clinical trials (Chapter 7). Indeed the study described in Chapter 8 demonstrates the value of this model in providing important information on the pharmacologically active dose range of a combined ET_A/ET_B receptor antagonist, L-753,037, *in vivo* at an early stage in its clinical development. BMS-193884 is an orally available biphenylsulfonamide derivative that selectively binds to recombinant human ET_A receptors with > 10 000 fold selectivity for human recombinant ET_A receptors over ET_B receptors (unpublished data[1]: Murugesan N *et al.* Biphenylsulfonamide endothelin receptor antagonists. 2. Discovery of 4'-Oxazolyl biphenylsulfonamides as a new class of potent, highly selective ET_A antagonists. Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 085343-5400, submitted for publication at time of submission of current manuscript).

We investigated the effects of BMS-193884 on the local vasoconstrictor response to ET-1 in healthy volunteers (Haynes, et al., 1996; Strachan, et al., 1998). In addition the systemic haemodynamic effects of BMS-193884 were investigated.

9.2 Methods

9.2.1 Subjects

28 healthy men, aged between 18 and 60 years, and within 15% of ideal body weight, were recruited to the study which was performed in the Clinical Research Centre at the Western General Hospital, Edinburgh with the approval of the local research ethics committee and the written informed consent of each subject. The investigations conformed with the principles outlined in the Declaration of Helsinki. All subjects attended for a pre-trial screening visit for full assessment of medical history and a physical examination. All subjects tested negative for HBsAg and for drugs of abuse. No subject received any medication, including over the counter medications, in the 2 weeks before each treatment period. All subjects abstained from alcohol for 48 hours and from food, caffeine containing drinks and tobacco for at least 7 hours before BMS-193884 and ET-1 administration. All studies were performed in a quiet room kept at a controlled temperature between 22-24°C.

9.2.2 Drugs

Intra-arterial administration

Endothelin-1 (Calbiochem, Nottingham, UK) was administered by continuous intra-arterial infusion for 60 min (Protocol 1) and 120 min (Protocol 2 & 3) at an infusion rate of 5 pmol/min, as described previously (Ferro, et al., 1997; Haynes, et al., 1996; Haynes and Webb, 1994), ET-1 administration was double-blind in Protocol 1 and single-blind in Protocols 2 & 3.

BMS-193884 (Bristol-Myers Squibb Pharmaceutical Research Institute, Moreton, Wirral, UK) was prepared as a crystalline free acid and suplied as a 5 mg/ml aqueous solution for infusion. In protocol 1, placebo (0.9% saline, Baxter Healthcare Ltd, Thetford, UK) or BMS-193884 (5 and 50 nmol/min) was administered double-blind by continuous intra-arterial infusion for 60 min, according to the study randomisation

schedule. Active intra-arterial infusion was followed by a 30 min washout infusion of saline 0.9%.

BMS-193884 inhibits $^{[125I]}$ET-1 binding to recombinant human ET_A receptors in the nanomolar and ET_B receptors in the micromolar range (K_iET_A 1.4 ± 0.1 nM, K_iET_B 18.8 ± 2 μM) (Unpublished data[1]: Murugesan N *et al.* Biphenylsulfonamide endothelin receptor antagonists. 2. Discovery of 4'-Oxazolyl biphenylsulfonamides as a new class of potent, highly selective ET_A antagonists. Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 085343-5400, submitted for publication at time of submission of current manuscript). Although it is not possible to quantify exact tissue concentrations during local intra-arterial infusion, both doses of BMS-193884 were estimated to be within a range selective for the ET_A receptor (5 nmol/min ≈100 nM, 50 nmol/min ≈1 μM, assuming arterial blood flow ≈50 ml/min). All dilutions were prepared in 0.9% saline (Baxter Healthcare Ltd) from sterile stock solutions on the day of the study.

Intra-arterial infusion

The brachial artery of the non-dominant arm was cannulated under local anesthesia (1% lidocaine; Astra Pharmaceuticals, Kings Langley, England) with a 27 SWG steel cannula (Cooper's Needle Works, Birmingham, UK) attached to a 16-gauge epidural catheter (Portex Ltd, Hythe, UK). Patency was maintained by infusion of saline via an IVAC P1000 syringe pump (IVAC Ltd, Basingstoke, UK). The total rate of intra-arterial infusions was kept constant at 1 ml/min throughout all studies. During co-infusion in Protocol 1 each agent was, therefore, infused at a rate of 0.5 ml/min.

Oral administration

BMS-193884 (Bristol-Myers Squibb Pharmaceutical Research Institute) was prepared as a crystalline free acid and supplied as a capsule dosage form for oral administration.

BMS-193884 (50, 100 or 200 mg) or matching placebo capsules (Bristol-Myers Squibb Pharmaceutical Research Institute) were administered orally in Protocols 2 and 3, according to the study randomisation schedule, administration was double-blind.

9.2.3 Measurements

Forearm Blood Flow

The response to brachial artery infusion was assessed in both forearms by measurement of blood flow using venous occlusion plethysmography, as described previously (Strachan, et al., 1998; Webb, 1995). Venous occlusion plethysmography was performed using a dual channel strain gauge plethysmograph (DE Hokanson Inc, Bellevue, WA, USA). Recordings of forearm blood flow were made over 3 min periods at 10 min intervals throughout intra-arterial infusions.

Systemic Hemodynamics

Hemodynamic recordings were made before (Protocols 2 & 3) and at 2 and 4 hours after (Protocol 3) oral drug administration, and then at regular intervals throughout the intra-arterial infusions (Protocols 1, 2 & 3).

Blood pressure and heart rate were measured in the non-infused arm using a well-validated semiautomated non-invasive method (Takeda UA 751 sphygmomanometer, Takeda Medical Inc., Tokyo, Japan.) (Wiinberg, et al., 1988). Blood pressure is presented as mean arterial pressure (MAP; diastolic blood pressure + 1/3 pulse pressure). During intra-arterial infusions, blood pressure was measured immediately after forearm blood flow measurements to avoid any effect of the venous congestion caused by this procedure on these measurements.

Cardiac output (CO) and stroke volume (SV) were recorded by a well validated non-invasive bioimpedance technique (NCCOM3; BoMed Medical Manufacturer Ltd,

Irvine, California, USA) (Thomas, 1992). Total peripheral vascular resistance (TPVR) was calculated as MAP divided by CO and expressed in arbitrary units.

Blood Sampling

Blood samples for assay of ET-1 and BMS-193884 and its metabolites were obtained, within 30 min of the start and at the end of the ET-1 infusion, via an 18 SWG cannula sited in the non-infused arm (Protocols 2 & 3). In brief, 10 ml samples were collected into sterile EDTA tubes (K3 EDTA, Vacutainer, Becton Dickinson Vacutainer Systems, Europe) centrifuged immediately at 1000 *g* for 15 min (BMS-193884 assay) or 20 min (ET-1 assay) and stored in plain tubes at -80°C prior to assay.

Endothelin-1 and big ET-1 were determined by standard radioimmunoassay (Peninsula Laboratories Europe), as described previously (Hand, et al., 1999). Concentrations of BMS-193884 and its metabolites, BMS-205868 and BMS-212442, were determined by a well validated standard liquid chromatography/mass spectrometry method (Unpublished data[2]: Eades DM. To develop an LC/MS/MS method for the quantitative determination of BMS-193884 and two of its metabolites in various matrices. AAPS Proceedings, 1997. AAPS Meeting, November 1, 1997).

Additional blood samples were taken at screening, on admission, prior to discharge and at the post trial physical examination for standard biochemistry and haematology assays. Routine urinalysis was carried out at screening and after the trial .

9.2.4 Study design

Protocol 1 - The local effects of intra-arterial infusion of ET-1 & BMS-193884

Twelve subjects were recruited to a double-blind, randomised 3 way crossover study. Subjects attended for 3 visits, each separated by 7 days. On separate occasions, in a random order, subjects received a 60 min intra-arterial infusion of ET-1 alone, BMS-193884 alone or co-infusion of ET-1 and BMS-193884. BMS-193884 was administered at a dose level of 5 nmol/min to 6 subjects in the first part of the study (Part A) and at a dose level of 50 nmol/min to 6 subjects in the second part of the study (Part B).

Blood pressure, heart rate, cardiac output and stroke volume were measured immediately before and at 15, 30 and 60 min during the intra-arterial infusions. Subjects attended the research centre on the morning of each study day and were discharged at least 5 hours after intra-arterial infusion.

Protocol 2 - The effect of oral administration of BMS-193884 systemic hemodynamics and the response to intra-arterial infusion of ET-1 at 12 hours

Eight subjects were recruited to a double-blind, randomised 4 way crossover study. All subjects attended for 4 visits, each separated by 7 days. The effect of intra-arterial infusion of ET-1 (5 pmol/min for 120 min) was assessed on separate occasions, 12 hours after oral administration of BMS-193884 (50, 100 or 200 mg) or matched placebo.

Blood pressure, heart rate, cardiac output and stroke volume were measured immediately before oral drug administration, 30 min before and immediately before intra-arterial infusion of ET-1, and at 30, 60 and 120 min after the start of the ET-1 infusion. Blood samples were taken from the non-dominant arm for ET-1 assay immediately before and 1 hour after the start of the ET-1 infusion and for

pharmacokinetic assay immediately before, and at the end of, the ET-1 infusion. Subjects attended the research centre at 4 pm on the evening before oral drug administration, stayed overnight and were discharged at least 5 hours after intra-arterial infusion the following day. BMS-193884 or placebo was administered at around 11 pm on the evening of admission. Intra-arterial infusion of ET-1 was timed to start at 10 hours after oral drug administration.

Protocol 3 - The effect of oral administration of BMS-193884 systemic hemodynamics and the response to intra-arterial infusion of ET-1 at 24 hours

Eight subjects were recruited to a double-blind, randomised 2 way crossover study. All subjects attended for 2 visits, each separated by 7 days. The effect of intra-arterial infusion of ET-1 (5 pmol/min for 120 min) was assessed on separate occasions, 24 hours after oral administration of BMS-193884 (200 mg) or matched placebo.

Blood pressure, heart rate, cardiac output and stroke volume were measured immediately before, 2 and 4 hours after oral drug administration, at 30 min before and immediately before intra-arterial infusion of ET-1, and at 30, 60 and 120 min after the start of the ET-1 infusion. Blood samples were taken from the non-dominant arm for ET-1 assay at 2 and 4 hours after oral dosing and immediately before and 1 hour after the start of the ET-1 infusion and for pharmacokinetic assay immediately before, and at the end of, the ET-1 infusion. Subjects attended the research centre at 9 pm on the evening before oral drug administration, were resident for 2 days and nights and were discharged at least 5 hours after intra-arterial infusion on day 2. BMS-193884 or placebo was administered at approximately 11 am on the morning after admission. Intra-arterial infusion of ET-1 was timed to start at 22 hours after oral drug administration.

Forearm blood flow studies (Protocols 1, 2 & 3)

Subjects rested recumbent throughout each forearm blood flow study in a quiet temperature-controlled room (23-25°C). Strain gauges and arm cuffs were applied and the brachial artery cannula was sited in the non-dominant arm. Saline (0.9%) was infused for at least 30 min, during which 3 measurements of forearm blood flow were made before the active intra-arterial infusion. Forearm blood flow recordings were made at 10 min intervals throughout.

9.2.5 Analysis

Forearm blood flow

Plethysmographic data listings were extracted from data files and forearm blood flows calculated for individual venous occlusion cuff inflations using a template spreadsheet (Excel 5.0; Microsoft Ltd, Wokingham, UK). Because wrist cuff inflation results in a transient forearm vasoconstriction, recordings made in the first 60 sec after wrist cuff inflation were not used for analysis. Blood flow in both forearms was obtained from the mean of the last 5 consecutive recordings of each measurement period. Baseline blood flow was taken as the last measurement during the saline infusion, before the start of the ET-1 infusion.

Forearm blood flow results are expressed as the percentage change from baseline in the ratio of blood flow between the infused and non-infused arms (Benjamin, et al., 1995; Strachan, et al., 1998; Webb, 1995). Data from the active intra-arterial infusion were examined by repeated-measures analysis of variance (ANOVA).

Systemic hemodynamics

Hemodynamic results are expressed as maximum placebo corrected percentage changes from baseline (Haynes, et al., 1996). Statistical analysis was performed on

untransformed data with cardiac function (bioimpedance method), heart rate and blood pressure data examined by repeated-measures analysis of variance (ANOVA).

Plasma ET-1

Plasma concentrations of ET-1 are represented as absolute change from predose (pg/ml) with statistical significance assessed by paired *t*-test.

Pharmacokinetic assay of BMS-193884 & metabolites

Plasma concentrations of BMS-193884, BMS-205868 and BMS-212442 are represented as absolute change from predose (ng/ml). No statistical analysis was performed on the data.

All results are expressed as mean ± standard error of the mean (SEM). Data analysis was performed using an Excel data analysis package (Excel 5.0; Microsoft Ltd, Wokingham, UK). For ANOVA comparisons, treatment and subject were the factors and the p values are for treatment comparisons over all timepoints. Statistical significance was taken at the 5% level.

9.3 Results

Twenty two subjects (mean age 37 ± 2 yrs), completed the study; 12 subjects completed Protocol 1, 7 subjects completed Protocol 2, and 8 subjects completed Protocol 3. Five subjects participated in both Protocol 1, in which they received only locally active doses of study drugs, and Protocol 3, with 6 - 12 weeks between their last study visit for Protocol 1 and their first dosing day for Protocol 3. Four subjects were withdrawn from Protocol 2; 1 subject was withdrawn as a result of adverse events judged to be unrelated to study drug administration, 3 subjects were withdrawn because of technical difficulties with arterial cannulation. Three of the subjects withdrawn from Protocol 2 were replaced.

There were no serious or unexpected adverse events and no significant changes in safety evaluations. Headache was the most commonly reported adverse event thought to be related to study drug administration with 23 reported events (from 16 subjects) on active treatment and 2 (from 2 subjects) on placebo treatment. Other events possibly related to active drug administration, but less frequently reported, were facial flushing (3 events, 3 subjects: 2 on active treatment), drowsiness (2 events, 2 subjects: 2 on active treatment) and nausea and vomiting (4 events, 3 subjects: 2 on active treatment).

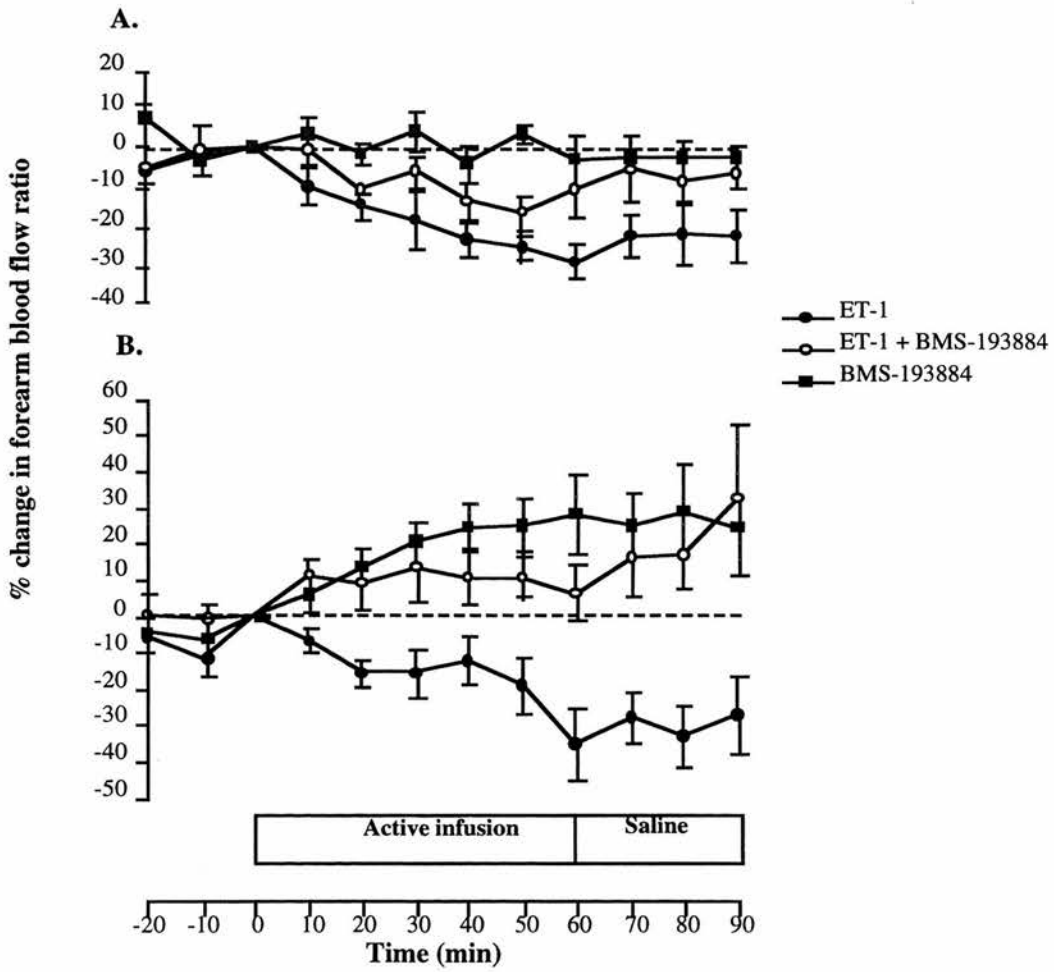
Protocol 1 - The local effects of intra-arterial infusion of ET-1 & BMS-193884

There was no significant change in blood pressure and heart rate, or in blood flow measured in the non-infused arm, at the end of intra-arterial infusions, confirming that any drug effects were confined to the infused arm (Table 9.1).

Forearm blood flow

There was significant local vasoconstriction, indicated by a reduction in the ratio of forearm blood flow, in response to intra-arterial infusion of ET-1 in both Part A and Part B of the study ($p<0.0001$ both parts) (Figure 9.1). Intra-arterial infusion of BMS-193884 (5 nmol/min) attenuated the constrictor response to ET-1 ($p<0.0001$) and had no significant vasodilator effect when infused alone ($p=0.7$) (Figure 9.1A). At the higher dose of 50 nmol/min, BMS-193884 abolished the constrictor response to ET-1 ($p<0.0001$) and caused vasodilatation when infused alone ($p=0.02$) (Figure 9.1B).

Figure 9.1 Response of forearm blood flow to local intra-arterial infusion of ET-1(5 pmol/min) alone, ET-1 co-infused with BMS-193884 (5 nmol/min, Part A; 50 nmol/min, part B) and BMS-193884 alone: ET-1, closed circles; BMS-193884 alone, closed squares; ET-1 + BMS-193884, open circles. Part A results shown in Figure 1A, Part B results shown in Figure 1B. Responses are expressed as mean % change in forearm blood flow ratio ± SEM. ET-1 caused significant forearm vasoconstriction, which was attenuated during co-infusion with BMS-193884 (5 nmol/min; p<, n=6 and 50 nmol/min; p<0.0001, n=6). BMS-193884 (50 nmol/min) caused local vasodilatation (25 ± 11%) when infused alone (p=0.02; n=6).



Protocol 2 - The effect of oral administration of BMS-193884 systemic hemodynamics and the response to intra-arterial infusion of ET-1 at 12 hours

There was no significant change in blood pressure and heart rate, or in blood flow measured in the non-infused arm, at the end of intra-arterial infusions, confirming that any drug effects were confined to the infused arm (Table 9.2).

Forearm blood flow

Forearm vasoconstriction to intra-arterial infusion of ET-1 was slightly attenuated after oral administration of BMS-193884 (200 mg) ($p<0.01$; Figure 9.2A). In contrast, there was no significant effect on ET-1 vasoconstriction after administration of BMS-193884 (50 or 100 mg; Table 9.2).

Systemic hemodynamics

Mean arterial pressure did not alter significantly after administration of BMS-193884 at any dose, with the exception of BMS-193884 50 mg (5 ± 6%, p=0.02; Table 9.3). There were small changes in cardiac function, indicative of systemic vasodilatation, after administration of BMS-193884 (200 mg) when compared with placebo. Although there was no significant change in stroke volume (1 ± 7%, p=0.3), cardiac output increased (11 ± 7%, p=0.01) and there was a reduction in total peripheral vascular resistance (-14 ± 9%, p=0.03) up until 12 hours after oral dosing with BMS-193884 (200 mg) (Figure 9.3). There was no significant change in any of the systemic hemodynamic parameters after administration of BMS-193884 100 mg (Table 9.3). In contrast, there were systemic changes after administration of BMS-193884 50 mg (stroke volume: -10 ± 4%, p=0.03; cardiac output: -8 ± 6%, p=0.003; total peripheral vascular resistance: 16 ± 9%, p=0.0004; Table 9.3). There were no significant systemic hemodynamic changes after placebo administration (Table 9.3).

Plasma ET-1

There was no significant change in plasma ET-1 concentrations for any of the treatments compared with placebo ($p>0.3$, at 10 and 11 hours; Table 9.4).

Pharmacokinetic assay of BMS-193884 & metabolites

Plasma concentrations of BMS-193884 and its metabolites, BMS-205868 and BMS-212442, increased dose dependently, were higher at 10 than 12 hours after administration (Table 9.5) and were not detected after placebo administration.

Protocol 3 - The effect of oral administration of BMS-193884 systemic hemodynamics and the response to intra-arterial infusion of ET-1 at 24 hours

There was no significant change in blood pressure and heart rate or in blood flow, measured in the non-infused arm, during intra-arterial infusion, confirming that any effects of intra-arterial infusion were confined to the infused arm (Table 9.2).

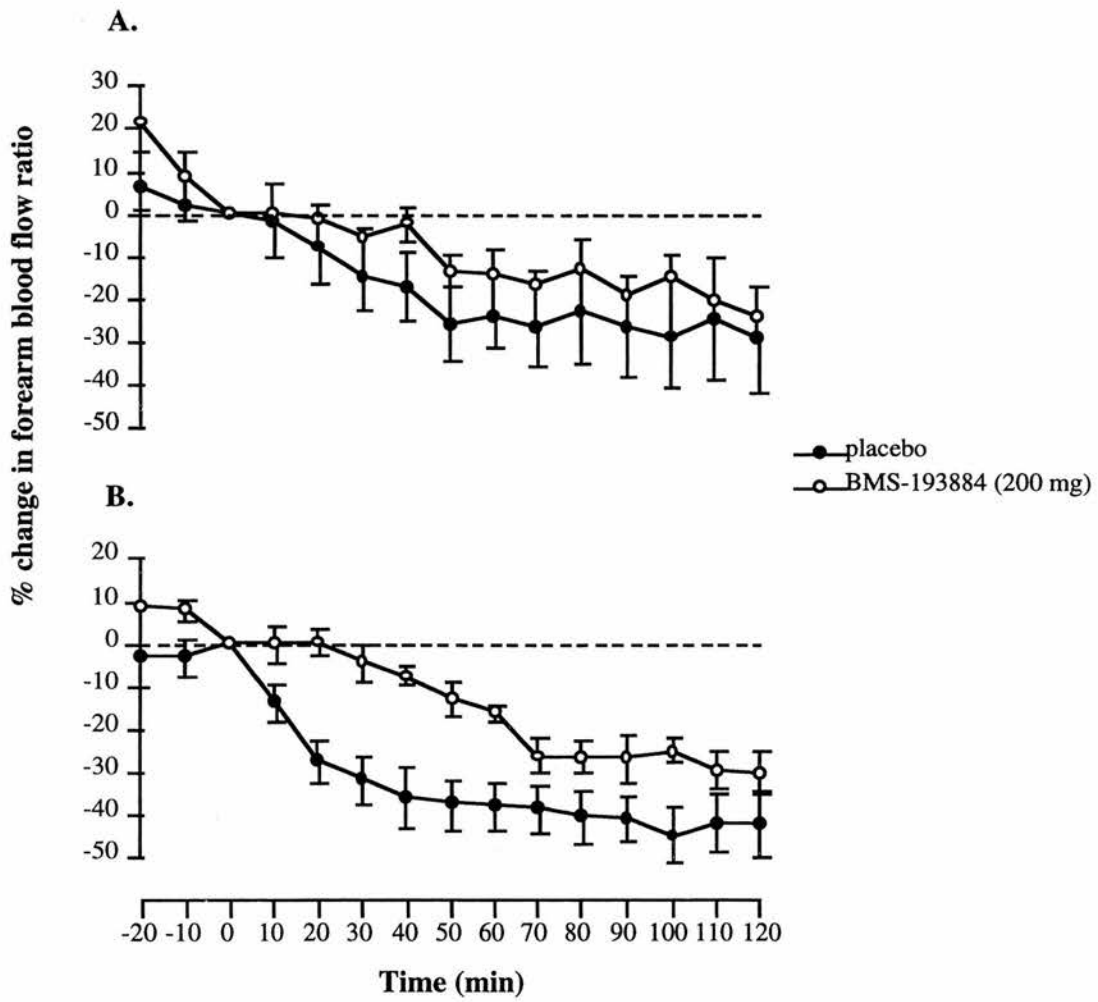
Forearm blood flow

There was significant vasoconstriction in response to intra-arterial infusion of ET-1 after placebo administration ($-42 \pm 5\%$, $p<0.0001$). The response to intra-arterial infusion of ET-1 was significantly attenuated after oral administration of BMS-193884 ($-30 \pm 8\%$, BMS-193884 vs $-42 \pm 5\%$, placebo; $p<0.0001$) (Figure 9.2B).

Table 9.2 Mean arterial pressure (MAP), heart rate (HR) and forearm blood flow (FBF) predose and at baseline and 120 min after the start of ET-1infusion, for Protocols 2 & 3. Values are mean ± SEM.

	PROTOCOL 2				PROTOCOL 3	
	placebo	**BMS-193884 50 mg**	**BMS-193884 100 mg**	**BMS-193884 200 mg**	**placebo**	**BMS-193884 200 mg**
MAP (mmHg)						
Basal	93 ± 3	94 ± 4	91 ± 4	92 ± 3	84 ± 2	88 ± 3
120 Mins	99 ± 5	98 ± 4	98 ± 4	95 ± 4	88 ± 2	93 ± 2
HR (bpm)						
Basal	54 ± 3	60 ± 4	63 ± 2	60 ± 4	57 ± 3	61 ± 3
120 Mins	58 ± 3	63 ± 5	61 ± 2	63 ± 4	54 ± 2	61 ± 3
FBF						
Control Arm (ml/100ml/min)						
Basal	2.4 ± 0.2	3.5 ± 0.3	3.0 ± 0.5	3.1 ± 0.3	3.2 ± 0.9	3.7 ± 0.6
60 min	2.9 ± 0.3	3.3 ± 0.4	2.8 ± 0.5	3.4 ± 0.3	3.6 ± 0.6	4.1 ± 0.6
Infused Arm (ml/100ml/min)						
Basal	3.0 ± 0.2	3.8 ± 0.4	3.7 ± 0.5	3.5 ± 0.3	2.8 ± 0.4	2.8 ± 0.4
60 min	2.4 ± 0.5	2.5 ± 0.3	2.7 ± 0.4	2.9 ± 0.2	2.0 ± 0.2	2.0 ± 0.2

Figure 9.2 Response of forearm blood flow to local intra-arterial infusion of ET-1 (5 pmol/min) 10 - 12 hours after oral administration of BMS-193884 (200 mg) or placebo, Protocol 2: placebo, closed circles; BMS-193884 (200mg), open circles; and 22 - 24 hours after oral administration of BMS-193884 (200 mg) or placebo, Protocol 3: placebo, closed circles; BMS-193884 (200 mg) open circles. Protocol 2 results shown in Figure 2A, Protocol 3 results shown in Figure 2B. Responses are expressed as mean % change in forearm blood flow ratio ± SEM. ET-1 caused significant forearm vasoconstriction, which was attenuated after oral dosing with BMS-193884 (200 mg), at 12 ($p<0.01$, n=7) and 24 hours ($p<0.0001$, n=8).



Systemic hemodynamics

Mean arterial pressure did not alter significantly after administration of BMS-193884 (Figure 9.3). There were small changes in cardiac function, indicative of systemic vasodilatation, after administration of BMS-193884 (200 mg) when compared with placebo: stroke volume and cardiac output increased (6 ± 7%, $p<0.001$ and 14 ± 7%, $p<0.0001$ respectively) and there was a reduction in total peripheral vascular resistance (-12 ± 7%, $p<0.0001$) 24 hours after oral dosing with BMS-193884 (200 mg) (Figure 9.3). There were no significant changes in systemic hemodynamics after placebo administration (Table 9.3).

Plasma ET-1

There was no significant change in plasma ET-1 concentrations after treatment with BMS-193884 compared with placebo ($p>0.1$, at 22 and 23 hours; Table 9.4).

Pharmacokinetic assay of BMS-193884 & metabolites

Plasma concentrations of BMS-193884, BMS-205868 and BMS-212442 were barely detectable (<10 ng/ml) at 24 hours and were not detected after placebo administration.

Figure 9.3 Systemic hemodynamic response (mean arterial pressure, heart rate, stroke volume, cardiac output, total peripheral vascular resistance) after oral administration of BMS-193884 (200 mg): Protocol 2, open circles; Protocol 3, closed circles. Responses are expressed as placebo corrected mean % change ± SEM. Oral administration of BMS-193884 (200 mg) caused systemic vasodilatation, indicated by a reduction in total peripheral vascular resistance at 12 (-14 ± 9%, p=0.03; n=7) and 24 hours (-12 ± 7%, p<0.0001; n=8).

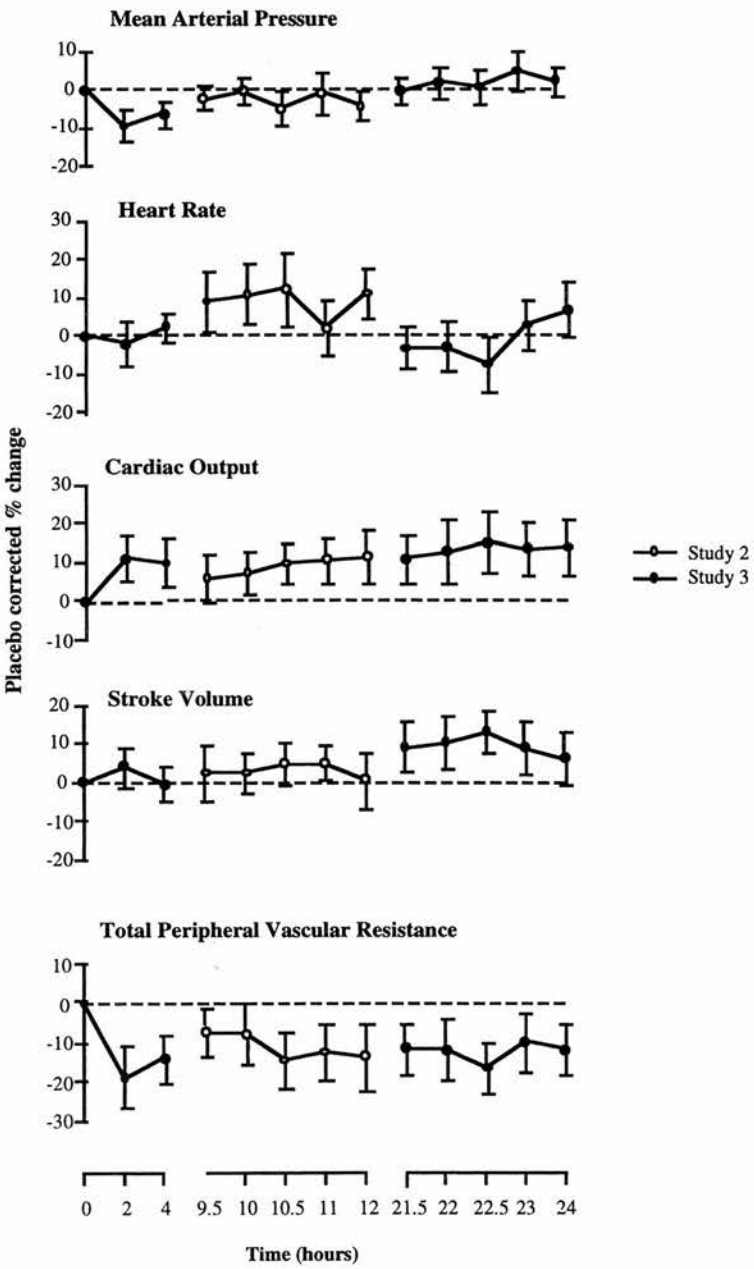


Table 9.3 Mean arterial pressure (MAP), heart rate (HR), cardiac output (CO), stroke volume (SV), total peripheral vascular resistance (TPVR) predose and at 12 hours (Protocol 2) and 24 hours (Protocol 3), after oral administration of placebo or BMS-193884. Values are mean ± SEM.

		PROTOCOL 2				PROTOCOL 3	
		placebo	**50 mg**	**100 mg**	**200 mg**	**placebo**	**200 mg**
MAP	**predose**	94 ± 4	89 ± 2	88 ± 3	92 ± 3	78 ± 2	80 ± 2
(mmHg)	**12 hours**	99 ± 5	98 ± 4	98 ± 4	95 ± 4		
	24 hours					88 ± 2	93 ± 2
HR	**predose**	57 ± 3	62 ± 4	64 ± 4	57 ± 3	57 ± 2	61 ± 3
(bpm)	**12 hours**	58 ± 3	63 ± 5	61 ± 2	63 ± 4		
	24 hours					54 ± 2	61 ± 3
CO	**predose**	6.8 ± 0.7	7.0 ± 0.5	7.0 ± 0.5	7.0 ± 1.0	5.9 ± 0.5	5.7 ± 0.4
(l/min)	**12 hours**	6.4 ± 0.5	6.1 ± 0.5	6.3 ± 0.5	7.2 ± 0.6		
	24 hours					5.7 ± 0.5	6.3 ± 0.4
SV	**predose**	115 ± 8	119 ± 8	116 ± 9	118 ± 16	105 ± 9	102 ± 8
(ml/min)	**12 hours**	112 ± 10	102 ± 9	102 ± 11	113 ± 12		
	24 hours					98 ± 10	98 ± 6
TPVR	**predose**	15 ± 2	13 ± 1	13 ± 1	15 ± 2	14 ± 1	15 ± 1
(arbitrary units)	**12 hours**	16 ± 2	17 ± 2	16 ± 2	14 ± 2		
	24 hours					17 ± 2	15 ± 1

Table 9.4 Mean plasma concentrations of ET-1 (pg/ml), predose, at 10 and 11 hours (Protocol 2) and 2, 4, 22 and 23 hours (Protocol 3) after oral administration of placebo or BMS-193884. Values are mean ± SEM.

Plasma ET-1	PROTOCOL 2				PROTOCOL 3	
(pg/ml)	placebo	50 mg	100 mg	200 mg	placebo	200 mg
predose	5.8 ± 0.7	5.6 ± 0.7	5.6 ± 0.7	5.7 ± 1.0	6.9 ± 0.7	5.9 ± 0.4
2 hours	-	-	-	-	6.1 ± 0.3	5.9 ± 0.7
4 hours	-	-	-	-	5.3 ± 0.3	6.4 ± 0.5
10 hours	5.6 ± 0.7	5.7 ± 0.6	6.1 ± 0.7	5.8 ± 0.7	-	-
11 hours	6.9 ± 1.2	6.1 ± 1.0	7.3 ± 1.3	6.7 ± 0.5	-	-
22 hours	-	-	-	-	5.7 ± 0.4	5.9 ± 0.4
23 hours	-	-	-	-	6.1 ± 0.8	5.4 ± 0.7

Table 9.5 Mean plasma concentrations of BMS-193884 and its metabolites, BMS-205868 and BMS-212442, at 10 and 12 hours after oral administration of BMS-193884 (50, 100 and 200 mg). Values are mean ± SEM. Placebo not shown.

	PROTOCOL 2								
Plasma concentrations (ng/ml)	**BMS-193884**			**BMS-205868**			**BMS-212442**		
	50 mg	**100 mg**	**200 mg**	**50 mg**	**100 mg**	**200 mg**	**50 mg**	**100 mg**	**200 mg**
10 hours	15 ± 2	111 ± 69	107 ± 24	4 ± 4	3 ± 3	1 ± 1	95 ± 11	170 ± 28	171 ± 37
12 hours	7 ± 1	33 ± 14	64 ± 16	<1	<1	1 ± 1	57 ± 8	89 ± 15	126 ± 34

9.4 Discussion

We have demonstrated vasoconstriction in the forearm vascular bed in response to infusion of locally active doses of ET-1 (5 pmol/min), consistent with previous results (Ferro, et al., 1997; Haynes, et al., 1996; Haynes and Webb, 1994; Strachan, et al., 1998). Using this model to assess the efficacy of endothelin receptor antagonists, we have shown that the ET_A receptor selective antagonist BMS-193884 attenuates ET-1 mediated constriction in the forearm vascular bed, when given locally or systemically, with the oral formulation demonstrating small effects for up to 24 hours.

Consistent with previous results with the ET_A receptor selective antagonist BQ-123 (Haynes and Webb, 1994), local forearm vasodilatation was demonstrated following intra-arterial infusion of BMS-193884 alone and, to a lesser degree, during co-infusion with ET-1, confirming the importance of endogenous ET-1 in the maintenace of vascular tone and indicating the therapeutic potential of these compounds as vasodilator treatments. Similarily, in addition to attenuating exogenous ET-1 mediated increases in forearm vascular tone, oral administration of BMS-193884 (200 mg) caused modest but significant systemic vasodilatation in healthy volunteers, similar to previous results with the mixed ET_A/ET_B receptor antagonist TAK-044 (Haynes, et al., 1996). This vasodilatation appeared to be dose-dependent and was clearer with observation over 24 rather than 12 hours. This may be related to the difference in the study design and the timing of drug administration and, therefore the difference in timing of predose and post dose assessments between protocols.

Plasma concentrations of ET-1 have been shown to increase after administration of ET_B receptor selective antagonists (Cowburn, et al., 1998; Fukuroda, et al., 1994; Gratton, et al., 1997; Strachan, et al., 1999) and mixed ET_A/ET_B receptor antagonists (Haynes, et al., 1996; Weber, et al., 1996) but not after adminstration of ET_A receptor selective antagonists (Ohnishi, et al., 1998; Spratt, et al., 1999), consistent with

clearance of ET-1 mediated by the ET_B receptor. In the current study we found no increase in plasma ET-1 concentrations after administration of BMS-193884, suggesting that its actions are predominantly mediated via the ET_A receptor in the current dose range.

As expected, plasma concentrations of BMS-193884 and its metabolites increased dose dependently and were higher at 12 hours than at 24 hours after oral drug administration, with levels at 24 hours barely detectable. Interestingly, although BMS-193884 and metabolite concentrations were almost undetectable at 23 hours post administration, attenuation of the vasoconstrictor response to ET-1 was still detected. This apparent dissociation between pharmacokinetic levels and pharmacodynamic effects has been described with other endothelin antagonist compounds (Haynes, et al., 1996). However, although reported plasma levels of BMS-193884 were less than 10 ng/ml (2 ± 1 ng/ml), the IC_{50} binding for human recombinant ET_A receptors is 1.4 nmol/L or 0.6 ng/ml. Therefore, although the plasma levels of the compound were reduced at 23 hours, the drug concentrations reached appeared to be active in terms of receptor blockade.

There were no serious or unexpected adverse events after administration of BMS-193884 in the current study. Headache, generally mild to moderate, was the most commonly reported event, and was effectively treated with paracetamol. Headaches are common with acute administration of endothelin receptor antagonists to healthy volunteers and most likely due to their vasodilator effects, perhaps through increased nitric oxide generation (Verhaar, et al., 1998).

A number of ET_A receptor selective and mixed ET_A/ET_B receptor antagonists are currently being investigated in clinical trials. It is unclear at present whether ET_A receptor selective or mixed ET_A/ET_B receptor antagonists will offer greater therapeutic

benefit. However, from our results in healthy volunteers (Strachan, et al., 1999; Verhaar, et al., 1998) and from results in animal models (Gratton, et al., 1997), it would appear that selective ET_A receptor antagonists may provide greater vasodilatation in some circumstances by allowing blockade of the constrictor effects of the ET_A receptor while preserving the vasodilator effects of the ET_B receptor. Indeed, in heart failure patients, selective ET_B receptor antagonism results in both local (Love, et al., 1996) and systemic vasoconstriction (Cowburn, et al., 1998). In contrast, in patients with hypertension vasodilatation has been described in response to selective ET_B receptor antagonism (Cardillo, et al., 1999). Therefore, in this case, mixed ET_A/ET_B receptor antagonists may be more effective. Further investigation in patient groups is required to assess the acute and chronic effects of selective ET_A receptor antagonists and mixed ET_A/ET_B receptor antagonists and to assess which of these approaches offers the greater therapeutic advantage in the clinical setting.

In summary, using the forearm vasoconstrictor response to exogenously applied ET-1 as a model (Ferro, et al., 1997; Haynes, et al., 1996; Haynes and Webb, 1994; Strachan, et al., 1998) we have shown that the ET_A receptor selective antagonist BMS-193884 is effective in blocking ET-1 mediated increase in vascular tone in healthy volunteers, with small effects evident 24 hours after oral adminstration. We have also shown modest systemic vasodilator effects after oral administration of BMS-193884, again evident 24 hours after oral adminstration of 200 mg, indicating its potential as a vasodilator treatment for further investigation in clinical trials. More pronounced effects may have been evident with higher dose levels. The current study provides further information on the *in vivo* activity and duration of effect of BMS-193884, this information may help in the selection of an appropriate dose range for future clinical trials.

Footnote

Unpublished data[1]: Murugesan N *et al.* Biphenylsulfonamide endothelin receptor antagonists. 2. Discovery of 4'-Oxazolyl biphenylsulfonamides as a new class of potent, highly selective ETA antagonists. Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 085343-5400, submitted for publication at time of submission of current manuscript.

Unpublished data[2]: Eades DM. To develop an LC/MS/MS method for the quantitative determination of BMS-193884 and two of its metabolites in various matrices. AAPS Proceedings, 1997. AAPS Meeting, November 1, 1997.

Chapter 10

Systemic blockade of the ET_B receptor increases peripheral vascular resistance in healthy men.

Systemic blockade of the endothelin-B receptor increases peripheral vascular resistance in healthy men. Strachan FE, Spratt JC, Wilkinson, IB, Johnston NR, Gray GA, Webb DJ. Hypertension 1999;33:581-585.

10.1 Introduction

The importance of endothelin-1 (ET-1) as a mediator of basal vascular tone *in vivo* in man has been described in earlier chapters, by demonstration of local (Haynes, et al., 1996; Haynes and Webb, 1994; Verhaar, et al., 1998 and Chapter 6) and systemic (Haynes, et al., 1996, and Chapter 8 and Chapter 9) vasodilatation in response to endothelin receptor antagonism. The potent vasoconstrictor effects of ET-1 (Clarke, et al., 1989; Yanagisawa, et al., 1988), combined with the increased plasma concentrations of ET-1 associated with cardiovascular diseases including heart failure (Pacher, et al., 1993) and renal failure (Koyama, et al., 1989), provide strong evidence to support a functional role for ET-1 in the development and maintenance of the increased peripheral vascular resistance associated with these conditions.

Local vasoconstriction to ET_B receptor agonists has been described in healthy volunteers (Hand, et al., 1999; Strachan, et al., 2000; Strachan, et al., 1995; and Chapter 3 and Chapter 4) and in patients with heart failure (Love, et al., 1996). However, more recently, vasoconstriction following local administration of the selective ET_B receptor antagonist BQ-788 (Ishikawa, et al., 1994) has been described in healthy volunteers (Verhaar, et al., 1998; Chapter 6) and in patients with heart failure (Love, et al., 2000). The results with antagonists are particularly important as they indicate that the endogenous effect of vascular ET_B receptor stimulation *in vivo* favours vasodilatation. Indeed, hypertension has been described following administration of systemic doses of the selective ET_B receptor antagonists A192621 in rats and BQ-788 in rabbits *in vivo*, as well as in rescued ET_B knockout mice (Gratton, et al., 1997; Webb, et al., 1998). The vasoconstrictor effects of ET_B antagonism may result from direct blockade of an endothelial ET_B receptor mediated dilator tone or indirectly from displacement of endogenously generated ET-1 to vasoconstrictor ET_A receptors, or as a result of reduced clearance of ET-1 by vascular ET_B receptors.

Confirmation of the balance of the vascular effects mediated by the ET_B receptor in different circumstances is important in understanding the physiology of the endothelin system, and in determining whether selective ET_A receptor antagonists or combined ET_A/ET_B receptor antagonists are likely to be more effective vasodilator agents in the clinical setting.

As a further step in understanding the contribution of the ET_B receptor to the maintenance of vascular tone *in vivo*, the systemic hemodynamic effects of BQ-788 in healthy male volunteers were investigated.

10.2 Methods

10.2.1 Subjects

Five healthy male subjects between 18 and 50 years of age were recruited to the study, under the standard conditions listed in Chapter 2.

10.2.2 Drugs

BQ-788 (Clinalfa AG, Laufelfingen, Switzerland) was used as a selective ET_B receptor antagonist, the dose range (3-300 nmol/min) used in the current study was selected from previous work investigating the local effects of BQ-788 in the forearm circulation (Chapter 10) and from a dose ranging pilot study in which 2 volunteers were studied at each dose level (data not shown), see Chapter 2.

BQ-788 was dissolved in physiological saline (0.9%; Baxter Healthcare, Ltd). Saline (0.9%; Baxter Healthcare, Ltd) was administered as placebo. BQ-788 and placebo were administered single blind and infused intravenously via an 18 SWG cannula sited in the left antecubital vein at a constant rate for 15 minutes. All solutions were prepared from sterile stock solutions on the day of the study.

10.2.3 Measurements

Hemodynamic recordings

Hemodynamic recordings were made at 10 minute intervals from 30 minutes predose until 1 hour following the start of the infusion, with an additional blood pressure measurement at 15 minutes corresponding with the end of the infusion, and then at 30 minute intervals until 2 hours and hourly until 4 hours following the start of the infusion.

Blood pressure and heart rate were recorded in duplicate at each timepoint, as described in Chapter 2, and averaged for each timepoint. Cardiac output and stroke volume were recorded by a well validated non-invasive bioimpedance technique (NCCOM3; BoMed Medical Manufacturer Ltd, Irvine, California, USA). As described in Chapter 2, parameters were corrected for body surface area and described as cardiac index (CI, l/min/m^2) and stroke index (SI, ml/m^2) (Haynes, et al., 1996).

Plasma ET-1 and Big ET-1

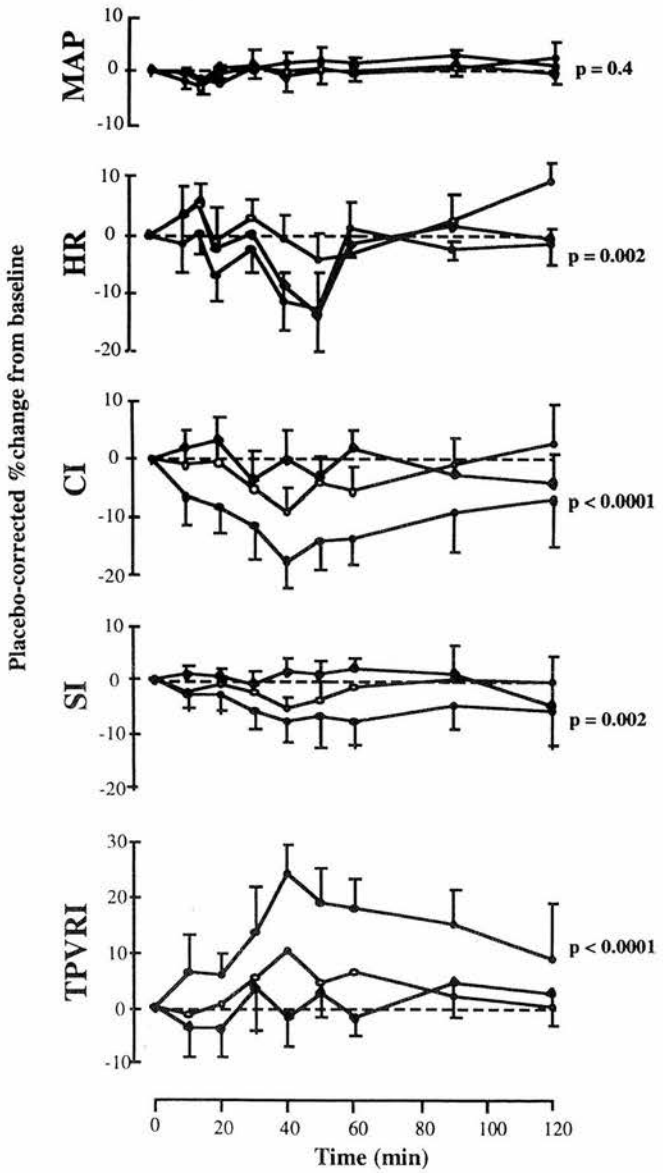
Blood samples were obtained for assay of ET-1 and big ET-1; predose and at 5, 15, 60 and 240 minutes post dose, via an 18 SWG cannula sited in the non-infused arm, as described in Chapter 2.

Blood samples were also taken on admission and prior to discharge for safety tests (sodium, potassium, creatinine, urea, alkaline phosphatase, gamma-glutamyl transpeptidase, hemoglobin and white cell count).

Table 10.1 Mean arterial pressure (MAP), heart rate (HR), cardiac output (CO), stroke volume (SV), total peripheral vascular resistance (TPVR) predose and at 60 min after administration of placebo or BQ-788. Values are mean ± SEM.

	Placebo	**BQ-788 (nmol/min)**		
		3	**30**	**300**
MAP (mmHg)				
Predose	80±3	81±2	79±2	80±2
60 min	80±3	81±2	79±2	82±1
HR (bpm)				
Predose	53±2	55±2	51±2	54±3
60 min	51±3	52±1	48±1	53±4
CI (l/min/m^2)				
Predose	2.6±0.2	2.8±0.2	2.7±0.2	2.9±0.3
60 min	2.6±0.2	2.8±0.2	2.5±0.2	2.5±0.4
SI (ml/min/m^2)				
Predose	49±3	51±3	52±4	53±3
60 min	48±2	51±3	51±4	49±5
TPVRI (Arbitrary units)				
Predose	31±2	30±2	29±2	29±2
60 min	32±1	30±2	32±2	35±4

Figure 10.1 Placebo corrected mean percentage change in hemodynamic parameters; mean arterial pressure (MAP), heart rate (HR), cardiac index (CI), stroke index (SI), total peripheral vascular resistance (TPVRI) following a 15 minute intravenous infusion of BQ-788 or saline placebo in five subjects: closed circles, BQ-788 (300 nmol/min); open circles, BQ-788 (30 nmol/min); shaded diamonds, BQ-788 (3 nmol/min). There was no change in MAP, but there was a reduction in HR, CI and SI, and an increase in TPVRI following administration of BQ-788 (300 nmol/min).



Plasma ET-1 and Big ET-1

There was no significant difference between predose plasma ET-1 concentrations for any of the treatments (range of baseline mean values 4.4-4.9 pg/ml). Plasma ET-1 concentration increased significantly following administration of BQ-788 (from 4.6±0.8 to 8.4±1.8 pg/ml at 15 minutes with 300 nmol/min, p=0.02) (Figure 10.2) but not during treatment with the lower doses of BQ-788 or placebo (Table 10.2). In contrast, concentrations of big ET-1 did not change significantly with treatment.

Figure 10.2 The change in plasma ET-1 concentrations (pg/ml) following a 15 minute intravenous infusion of BQ-788 or saline placebo in five subjects: solid columns, BQ-788 (300 nmol/min); shaded columns, BQ-788 (30 nmol/min); hatched columns, BQ-788 (3 nmol/min); open columns, placebo. Plasma ET-1 concentrations increased significantly following administration of BQ-788 (300 nmol/min).

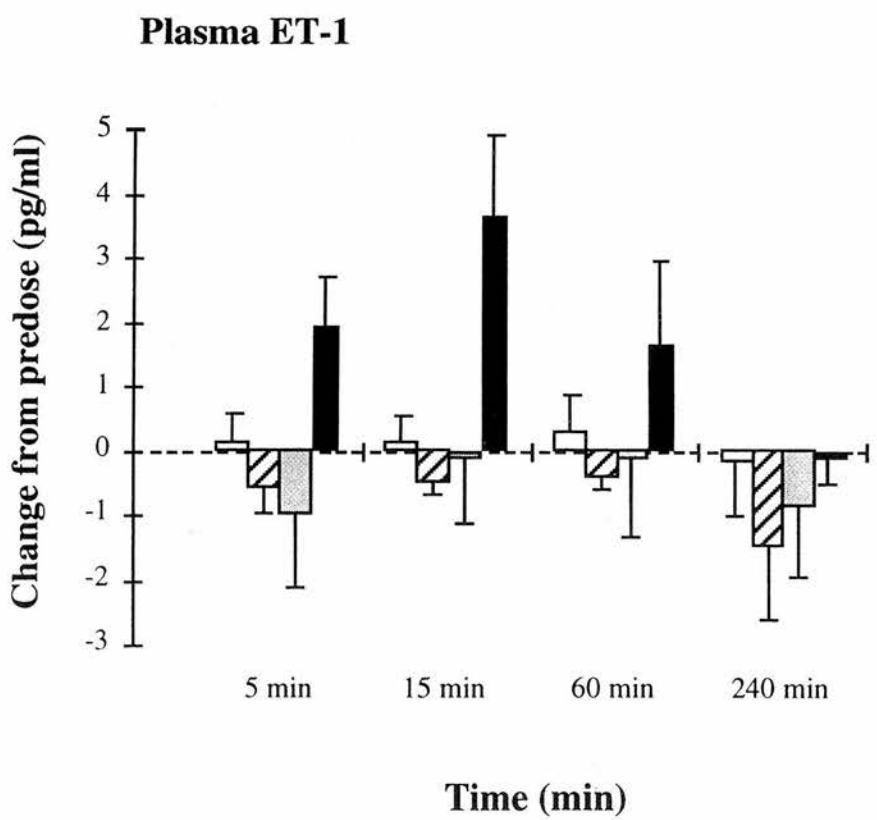


Table 10.2 Mean plasma concentrations of ET-1 (pg/ml), predose and at 60 min after administration of placebo or BQ-788. Values are mean ± SEM.

	Placebo	BQ-788 (nmol/min)		
		3	30	300
Plasma ET-1 (pg/ml)				
Predose	4.4±1.0	4.8±0.8	5.2±1.0	4.6±0.8
60 min	4.4±0.5	4.4±0.7	4.8±1.0	6.3±1.4

10.4 Discussion

Substantial systemic vasoconstriction, associated with a reduction in heart rate and cardiac index but no change in blood pressure, has been demonstrated in the current study in response to administration of the selective ET_B receptor antagonist BQ-788 in healthy men. Consistent with earlier work in the forearm circulation (Verhaar, et al., 1998; Chapter 6) these observations are highly suggestive of the overall effect of endogenous ET_B receptor mediated vascular tone favouring vasodilatation. An alternative explanation for the hemodynamic effects is that BQ-788 is directly negatively chronotropic and that peripheral effects are indirect. However, this is unlikely given the local vasoconstriction following local infusion of BQ-788 (Verhaar, et al., 1998; Chapter 6), and the lack of evidence of an important positive chronotropic and inotropic role of the cardiac ET_B receptor (Zhu, et al., 1997). Although peripheral resistance was substantially increased, blood pressure was unaffected because of a decrease in heart rate which was probably reflex in origin. Increases in plasma ET-1,

but not big ET-1, concentrations were also demonstrated following ET_B receptor blockade, consistent with reduced clearance of ET-1 by the ET_B receptor (Fukuroda, et al., 1994). All of these effects were prominent with BQ-788 at the highest dose but were not clearly seen at lower doses.

The vasoconstrictor effects of ET_B receptor antagonism may result directly from blockade of the vasodilator effects of the endothelial ET_B receptor or indirectly from displacement of endogenously generated ET-1 from ET_B receptors to unoccupied ET_A receptors. It is unlikely that these effects are mediated by non-selective ET_A/ET_B receptor blockade because they are the opposite of those found with selective ETA receptor antagonists in healthy subjects (Spratt, et al., 1999) and patients with heart failure (Cowburn, et al., 1998), and of those found with combined ET_A/ET_B receptor antagonists in healthy subjects (Haynes, et al., 1996). Clearly, the indirect effects of ET-1 on ET_A receptors are more relevant with administration of selective ET_B antagonists than with non-selective ET_A/ET_B receptor antagonists, because in this latter situation the constrictor ET_A receptor is also blocked. Indeed, vasodilator effects have been demonstrated with both selective (Cowburn, et al., 1998; Haynes and Webb, 1994; Verhaar, et al., 1998) and non-selective (Haynes, et al., 1996; Kiowski, et al., 1995) endothelin receptor antagonists in humans and the non-selective ET_A/ET_B antagonist, bosentan, has also been shown to effectively lower blood pressure in patients with hypertension (Krum, et al., 1998). However, direct comparison of the effects of selective and non-selective endothelin receptor antagonism will be important in assessing the relative contribution of each receptor subtype to the vascular effects of ET-1.

Forearm vasodilatation in response to local ET_A receptor antagonism with BQ-123 has been demonstrated previously (Haynes, et al., 1994; Verhaar, et al., 1998; Berrazueta, et al., 1997; Chapter 6]. In the presence of BQ-788 in healthy volunteers

this effect was attenuated (Verhaar, et al., 1998; Chapter 6), suggesting that the overall effect of vascular ET_B receptor stimulation by endogenous ET-1 is vasodilatation. This attenuation of BQ-123 mediated vasodilatation by BQ-788 suggests that the vasoconstrictor effect of ETB receptor blockade is not mediated by displacement of ET-1 onto the ET_A receptor, but is due to direct blockade of ET_B mediated vasodilator tone. Using a 'nitric oxide clamp' technique, it has also been shown that the vasodilator response to BQ-123 is in part mediated by nitric oxide (Verhaar, et al., 1998) and, therefore, probably mediated by the endothelial ET_B receptor. The dilator response to high dose infusion of ET_B receptor selective agonist also appears to be mediated through generation of nitric oxide (Chapter 5). Loss of endothelial cell ET_B mediated vasodilator tone may occur in cardiovascular diseases, such as essential hypertension and hypercholesterolaemia, in which there is associated endothelial dysfunction (Casino, et al., 1995; Panza, et al., 1995). Here, because of a reduced capacity for ET_B receptor mediated, nitric oxide dependent dilatation, selective ET_A receptor antagonists may be less effective. Indeed, vasodilatation has been described in response to BQ-788 in patients with hypertension (Cardillo, et al., 1999), indicating that in this patient group, the dilator effects of the ET_B receptor may be less important than the constrictor effects.

In summary, the results of the current study demonstrate systemic vasoconstriction in response to acute ET_B receptor blockade with the selective ET_B receptor antagonist BQ-788 in healthy men *in vivo*, indicating that the predominant endogenous effect of stimulating vascular ET_B receptors is vasodilatation. One exciting possibility is that tonic endogenous ET-1 release, acting via the endothelial ET_B receptor, is responsible for the physiological basal release of nitric oxide. This now needs to be addressed in clinical studies. Further investigation of the influence of ET_B receptor antagonism on the sympathetic nervous system and renal function are also warranted. In addition, direct comparison of the effects of chronic administration of selective ET_A and

combined ET_A/ET_B receptor antagonists are required in patients with cardiovascular disease, with and without endothelial dysfunction, in order to confirm which of these approaches is likely to be more effective in the clinical setting.

Chapter 11

General discussion

11.1 Summary

The role of the endothelin system in the maintenance of vascular tone has been investigated in the current series of clinical studies, using local and systemic measures of vascular tone. In particular, the relative contribution of the ET_A and ET_B receptor towards ET-1 mediated vascular tone was investigated.

11.1.1 ET_B mediated constriction in capacitance vessels

Constriction in capacitance and resistance vessels was demonstrated in response to locally active infusion of the non-selective ET receptor agonist ET-1 and the ET_B receptor selective agonists SFTX6c and BQ-3020, supporting a potential role for the ET_B receptor in ET-1 mediated vasoconstriction. In addition, the demonstration of vasoconstriction in response to SFTX6c and the structurally distinct agonist BQ-3020 addressed concerns over the validity of SFTX6c as a pharmacological probe for the ET_B receptor in clinical studies.

Constriction to ET-1 but not SFTX6c was attenuated during co-infusion of the ET_A receptor selective antagonist BQ-123 in the hand vein. In contrast, constriction to SFTX6c but not ET-1 was attenuated during co-infusion of the ET_B receptor selective antagonist BQ-788. These results suggest that while the constrictor effects of ET_B receptor agonists are mediated through the ET_B receptor, the ET_B receptor does not play a direct role in ET-1 mediated vasoconstriction.

11.1.2 ET_B mediated vasoconstriction and vasodilatation in resisitance vessels

Consistent with the effects described in the hand vein, local vasoconstriction in response to locally active infusion of the non-selective ET receptor agonist ET-1 and the ET_B receptor selective agonists SFTX6c and BQ-3020 was demonstrated in forearm resistance vessels. In addition to the vasoconstrictor effects of ET_B receptor

agonists, the vasodilator response to high dose infusion of the ET_B agonist SFTX6c was investigated. The vasodilator effect was blocked by infusion of the ET_B receptor selective antagonist BQ-788 and the nitric oxide synthase inhibitor L-NMMA, but not by aspirin or noradrenaline. These results indicate that this vasodilatation was mediated by the ET_B receptor, largely through nitric oxide, but not by prostacyclin, generation. In this study, the constrictor response to SFTX6c was not affected by infusion of BQ-788.

11.1.3 Local effects of endothelin receptor antagonists on vascular tone

Local vasoconstriction was demonstrated in response to locally active infusion of the ET_B receptor selective antagonist BQ-788. Interestingly, both ET_A receptor selective and combined ET_A/ET_B receptor antagonism resulted in significant forearm vasodilatation. However, the local vasodilator effects of the ET_A receptor selective antagonist BQ-123 were attenuated in the presence of the selective ET_B receptor antagonist BQ-788. These results confirm the importance of ET-1 in the maintenance of vascular tone and indicate that, in healthy blood vessels at least, the vasodilator effects of non-selective ETA/ETB receptor antagonism are less than those of ET_A receptor selective antagonism.

11.1.4 Systemic effects of endothelin receptor antagonists

The forearm vasoconstrictor response to intra-arterial infusion of ET-1 was assessed in detail and, using this response as a model, a pharmacologically active dose range for the non-selective ET_A/ET_B receptor antagonist MSD L-753,037 and the ET_A receptor selective antagonist BMS-193884 was identified. Both antagonists were effective in blocking ET-1 mediated vasoconstriction and also demonstrated modest but significant systemic vasodilator effects.

In a final evaluation of ET_B receptor function, the systemic effects of the ET_B receptor selective antagonist BQ-788 were assessed and significant systemic vasoconstriction demonstrated, with an increase in peripheral vascular resistance, and a reduction in cardiac index and stroke index. These results, in the systemic circulation, confirm the effects of locally active infusion of BQ-788, and provide further evidence that the balance of effects at the ET_B receptor in healthy blood vessels favours vasodilatation.

Administration of the non-selective ET_A/ET_B receptor antagonist MSD L-753,037 or the ET_B receptor selective antagonist BQ-788 resulted in significant increases in plasma concentrations of ET-1, suggesting activity at the ET_B clearance receptor. In contrast, there was no increase in plasma concentrations of ET-1 following administration of the ET_A receptor antagonist BMS 193884.

The results of these investigations have provided valuable information on the relative contribution of the ET_A and ET_B receptors to the physiological effects of ET-1 in the vasculature. Despite demonstration of vasoconstriction in response to ET_B receptor agonists, the predominant effect of the vascular ET_B receptors in healthy human blood vessels appears to be vasodilatation. However, it is important to acknowledge the potential constrictor effects of the increased plasma ET-1 concentrations associated with ET_B receptor antagonism. Further assessment is required both in healthy volunteers and in patients with cardiovascular disease.

In addition to the assessement of the relative contribution of the ET receptors in the mediation of vascular tone, the series of studies described in this thesis has developed and characterised a series of tools for future clinical studies.

11.2 Current research

Many endothelin receptor antagonists have progressed to clinical studies in healthy volunteers and in patient groups. However, as yet, none have been licensed for clinical use. Much of the current clinical research has focussed on cardiovascular disease.

11.2.1 Endothelin and vascular tone

The vasodilator effects of endothelin receptor antagonists have been confirmed by a number of investigators in healthy volunteers (Haynes, et al., 1996; Spratt, et al., 1999; Strachan, et al., 2000; Verhaar, et al., 2000) and in patients with cardiovascular disease (Cardillo, et al., 1999; Cowburn, et al., 1998; Love, et al., 2000). In addition assessment of the forearm blood flow response to intra-arterial infusion of ET-1, as described in Chapter 7, has allowed identification of pharmacologically active dose ranges and duration of effect for endothelin receptor antagonists in early phase I clinical trials (Haynes, et al., 1996; Verhaar, et al., 2000).

The constrictor effects of ET_B receptor selective agonists have been confirmed following local infusion of the endogenous ET_B receptor selective agonist, ET-3, in the dorsal hand vein and forearm resistance vessels of healthy volunteers (Ferro, et al., 2000). In addition, systemic vasoconstriction has been described following infusion of ET-3 in patients with heart failure (Cowburn, et al., 1999). However, local (Love, et al., 2000) and systemic (Cowburn, et al., 1998) vasoconstriction have also been described in patients with heart failure following infusion of the ET_B receptor selective antagonist BQ-788. These results suggest that, although the ET_B receptor may mediate vasoconstriction directly, the endogenous effects of the ET_B receptor in patients with heart failure appear to favour vasodilatation. In contrast, in patients with hypertension, vasodilatation has been described in response to infusion of BQ-788 (Cardillo, et al., 1999), indicating that, in this patient group, the endogenous effects of the ET_B receptor favour vasoconstriction. The effects described in hypertension may be due to the

recognised endothelial dysfunction in patients with hypertension and consequent reduction in capacity for endothelial ET_B receptor mediated vasodilatation. However, the dose used in this study (50 nmol/min) is higher than those used in the studies described in Chapters 4, 5 and 6 (1 nmol/min) and vasodilatation could result from non-selective effects at the ET_A receptor in this dose range. It is also important to recognise that the vasoconstrictor effects of ET_B receptor antagonism could result from increased plasma ET-1, as a result of reduced clearance at the ET_B receptor, acting at the unopposed ET_A receptors.

11.2.2 Endothelin: cardiac effects

Endothelin-1 is synthesized, stored and released in the human heart and exerts both indirect effects, through changes in coronary vascular tone, and direct effects on cardiac muscle (Russell, et al., 2000; for review). In contrast to results in rat models previously described (Garjani, et al., 1995), ET-1 induced arrhythmias in isolated human cardiac tissue were reduced in the presence of combined ET_A/ET_B receptor blockade and not in the presence of ET_A receptor selective blockade (Burrell, et al., 2000). A positive inotropic role for the ET_A receptor has been suggested (MacCarthy, et al., 2000). Further investigation of the endogenous effects of ET-1 on the contractility of the heart is required.

11.2.3 Endothelin and renal function

The potential therapeutic benefits of endothelin receptor antagonists and the endogenous effects of ET-1 have been investigated in healthy volunteers using selective and combined endothelin receptor antagonists. Following administration of the non-selective ET_A/ET_B receptor antagonist SB 209670, there was a modest increase in effective renal plasma flow (ERPF) indicating that endogenous ET-1 contributes to resting vascular tone within the kidney. In contrast, selective ET_B receptor antagonism with BQ-788 resulted in an increase in renal vascular resistance in healthy volunteers

and in patients with renal disease, indicating that the ET_B receptor in the renal vasculature favours vasodilatation (Goddard, et al., 2000) consistent with studies in systemic circulation (Strachan, et al., 1999).

In contrast to animal models, there were no changes in urinary sodium excretion following combined or selective ET receptor blockade (Freed, et al., 1999), indicating that ET-1 does not contribute significantly to sodium transport in resting conditions in healthy volunteers.

11.2.4 Endothelin receptor antagonists: clinical results

Endothelin receptor antagonists are currently in clinical development, and have shown potential therapeutic benefits. Although long term clinical studies are ongoing, the results of these studies are not currently available

Heart failure

Previous studies have demonstrated additional vasodilator benefits of endothelin receptor antagonists in patients with heart failure in the presence of existing treatment with ACE inhibitors (Kiowski, et al., 1995). Recent clinical studies confirmed the potential of ET receptor antagonists as vasodilator treatments in heart failure (Cowburn, et al., 1998; Givertz, et al., 2000; Love, et al., 2000; Miyauchi and Goto, 1999; Spieker, et al., 2000). The relative benefits of combined ET_A/ET_B and ET_A receptor selective antagonists are currently being investigated (Bauersachs, et al., 2000; Mulder, et al., 2000; Ohnishi, et al., 1998). Although systemic vasoconstriction has been described in response to the ET_B receptor selective agonist ET-3 (Cowburn, et al., 1999), the overall balance of effects at the ET_B receptor appears to favour vasodilatation (Cowburn, et al., 1998; Love, et al., 2000), indicating that ET_A receptor selective antagonists may offer greater therapeutic benefit as vasodilators. Indeed, upregulation of ET_A receptors in failing human myocardium has been described (Zolk,

et al., 1999) and, in a rat model, ET_A receptor selective antagonism shows greater improvement in endothelial vasomotor dysfunction than combined ET_A/ET_B receptor antagonism (Bauersachs, et al., 2000). In a dog model of heart failure, both combined ET_A/ET_B and ET_A receptor selective antagonism offered similar haemodynamic benefits but the ET_A receptor selective antagonist showed greater benefits in renal function (Ohnishi, et al., 1998), in this case, providing an additional benefit over combined ET_A/ET_B receptor antagonism. Interestingly, in the same study, plasma aldosterone concentrations were reduced following combined ET_A/ET_B receptor antagonism. This may be of benefit in long term treatment of heart failure by preventing volume retention by suppressing aldosterone secretion. Despite the differences described between combined and selective receptor antagonism, a recent long term study in a coronary ligation rat model, chronic administration of both ET_A receptor selective and combined ET_A/ET_B receptor antagonists improved systemic haemodynamics to a similar degree (Mulder, et al., 2000). However, combined ET_A/ET_B receptor blockade reduced heart rate, which may be of benefit in long term treatment of heart failure.

These data support the use of ET receptor antagonism in the treatment of heart failure. Further investigation of the long term benefits of ET receptor antagonism and comparison of the relative benefits of combined and selective ET receptor antagonists is required.

Hypertension

Endothelin receptor antagonism may also be of benefit in the treatment of hypertension. Treatment with the combined ET_A/ET_B receptor antagonist bosentan has been shown to lower blood pressure in patients with essential hypertension (Krum, et al., 1998) and local vasodilatation has been described, in patients with hypertension, following acute infusion of the ET_A receptor selective antagonist BQ-123 and co-infuson of BQ-123 and the ET_B receptor selective antagonist BQ-788 (Cardillo, et

al., 1999). Interestingly, local vasodilatation was described in patients with hypertension following infusion of BQ-788 alone (Cardillo, et al., 1999) and contrasts with the vasoconstriction described in healthy volunteers (Cardillo, et al., 1999; Strachan, et al., 1999; Verhaar, et al., 1998) and in patients with heart failure (Love, et al., 2000). This seems to indicate that, in patients with hypertension, the balance of effects of the ET_B receptor favours vasoconstriction. This may result from the endothelial dysfunction described in this patient group and their reduced capacity for nitric oxide dilatation (Panza, et al., 1995). However, it may result from non-selective effects of BQ-788 in the dose range used.

Interestingly, vascular smooth muscle cell ET_B receptors have been shown to be more abundant in human atherosclerotic lesions (Fan, et al., 2000), indicating that here ET_B receptor mediated vasoconstriction may be more important.

Renal failure

ET receptor antagonists have demonstrated potential clinical benefits in the treatment of renal failure (Strachan and Webb, 1998). Recent studies have confirmed this potential in healthy volunteers (Freed, et al., 1999), and in patients with renal disease (Goddard, et al., 2000). However, despite evidence to suggest involvement of ET-1 in the pathophysiology of radiocontrast nephrotoxicity and potential therapeutic benefits of ET receptor antagonism in an animal model (Cantley, et al., 1993), a recent study described exacerbation of radiocontrast nephrotoxicity following combined ET_A/ET_B receptor antagonism in patients undergoing cardiac angiography (Wang, et al., 2000). Selective ET_A receptor antagonists may be of benefit for this indication as ET_B mediated vasodilatation and ET-1 clearance would be preserved. Further investigation is required to investigate appropriate therapeutic approaches in renal disease.

Myocardial infarction

Plasma ET-1 concentrations are increased following myocardial infarction and are strongly related to mortality (Omland, et al., 1994) and endothelin receptor antagonists have been shown to reduce expression of molecular markers of failing myocardial function (Sakai, et al., 2000). Upregulation of smooth muscle ET_B receptors that modulate ET_A mediated constriction have been described following myocardial infarction in a rat model (Gray, et al., 2000). Therefore, it is possible that administration of ET_A receptor selective antagonists may be of more benefit in reducing constriction to increased plasma concentrations of ET-1 if myocardial infarction progresses to heart failure (Gray, et al., 2000).

Subarachnoid haemorrhage

A therapeutic role for ET_B receptor selective antagonism has been described in reducing cerebral vasospasm in a rabbit subarachnoid haemorrage model (Zucarello, et al., 1998). However, the systemic haemodynamic effects of ET_B receptor selective antagonists (Cowburn, et al., 1998; Strachan, et al., 1999) must also be considered if this approach were to be considered in a clinical setting.

11.3 Future directions

11.3.1 The role of the ET_B receptor in ET-1 mediated vascular tone

Although the results of the studies described in Chapter 3, Chapter 4 and Chapter 5 indicate that the ET_B receptor mediates constriction under direct stimulation from ET_B receptor agonists, the functional significance of this constriction remains unclear. Indeed, the vasoconstrictor effects of ET_B receptor antagonists in healthy volunteers appear to contradict a direct role for ET_B mediated constriction in ET-1 mediated

vascular tone. These studies require co-infusion of agonists and antagonists and their results can be difficult to interpret due to the additive effects of each agent on vascular tone and possible local increases in ET concentration following antagonism at the ET_B receptor. Therefore, the relative contribution of the ET_A and the ET_B receptor to the constrictor and dilator effects of ET receptor agonists described in Chapter 3 and Chapter 5, may be clearer if systemic administration of ET receptor antagonists was combined with local infusion of ET receptor agonists, similar to the studies described in Chapter 8 and Chapter 9.

Future studies investigating the dilator and constrictor effects of local infusion of ET_B receptor selective agonists following systemically active selective and combined ET_A/ET_B receptor antagonism would help address these difficulties, and also provide a cleaner model with which to assess the role of each receptor in the constrictor effects described with ET_B receptor agonists.

11.3.2 Combined ET_A/ET_B versus selective ETA receptor antagonism in cardiovascular disease

Recent research has focussed on the potential clinical benefits of endothelin receptor antagonists in animal models of cardiovascular disease and, latterly, in patient groups. However, few studies have compared directly the effects of selective ET_A and combined ET_A/ET_B receptor antagonists. Further investigation of the integrated cardiovascular effects of selective and non-selective ET receptor antagonists is required to confirm which approach will be the more effective in the clinical setting.

The results of the studies described in this thesis, in particular in Chapter 10, have assisted the development of models with which to assess the effects of ET receptor antagonists in humans *in vivo*. Indeed, using this methodology, studies investigating

the acute effects of ET receptor antagonists in patients with heart failure and in patients with renal disease are ongoing in our research group. Direct comparison of the integrated effects of endothelin receptor antagonists in cardiovascular disease, both during acute and chronic administration of combined and selective ET receptor antagonists are required.

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APPENDIX

A novel *S*-nitrosothiol (RIG200) causes prolonged relaxation in dorsal hand veins with damaged endothelium

Background: **Reduced nitric oxide bioavailability caused by endothelial dysfunction or damage is a contributory factor in the initiation and progression of a number of cardiovascular diseases. Delivery of exogenous nitric oxide is an attractive therapeutic option, but current agents lack selectivity for areas of endothelial damage. We tested the hypothesis that a novel nitric oxide donor drug, *N*-(*S*-nitroso-*N*-acetylpenicillamine)-2-amino-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-glucopyranose [RIG200], which has selective effects in endothelium-denuded isolated arteries in vitro, would exert similar effects in dorsal hand veins with experimentally damaged endothelium in vivo.**

Methods: **Venodilator responses to sodium nitroprusside and RIG200 were compared in two groups of healthy volunteers (age range, 18 to 63 years; n = 7 for each group) in norepinephrine 70% maximum effective concentration (EC_{70}) preconstricted hand veins with use of the Aellig technique. In this double-blind study, subjects were randomly assigned to receive either sodium nitroprusside or RIG200 (infusions of 0.06 and 6 nmol/min into the hand vein) before and 2 days after 15 minutes of local venous irrigation with distilled water. Endothelial function was assessed in all subjects on both visits with use of the endothelium-dependent vasodilator acetylcholine (1 nmol/min).**

Results: **Irrigation of hand veins with distilled water abolished endothelium-dependent dilatation in response to acetylcholine in both study groups (n = 14) but did not affect the amplitude or duration of responses to the conventional nitric oxide donor sodium nitroprusside ($P = .87$; n = 7). However, responses to RIG200 were significantly prolonged during the washout phase (30 minutes) in veins after water irrigation ($P = .02$; n = 7).**

Conclusion: **These studies confirm that RIG200 has prolonged effects in veins with damaged endothelium, a characteristic that might be exploited therapeutically to target nitric oxide delivery to damaged blood vessels. (Clin Pharmacol Ther 2000;68:75-81.)**

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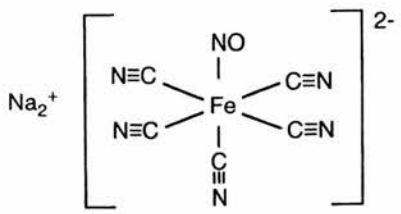
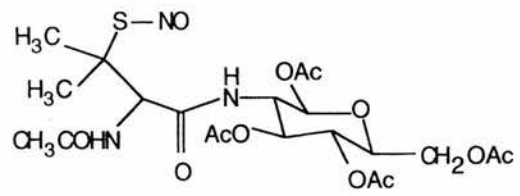
The vascular endothelium plays a pivotal role in regulating blood vessel structure and function through release of a number of vasoactive mediators. One of these mediators is nitric oxide, which dilates blood vessels[1-3] and inhibits platelet aggregation,[4,5] platelet adhesion,[6,7] smooth muscle proliferation,[8] and monocyte adhesion.[9] Reduced nitric oxide production from vessels with damaged or dysfunctional endothelium causes a predisposition to vasospasm and thrombosis[10] and has been implicated as an early step in atherogenesis.[11] Morphologic studies have also indicated that small areas of endothelial cell erosion are common over established coronary plaques.[12,13] Focal endothelial denudation associated with atheromatous plaques may



Sodium nitroprusside



N-(S-nitroso-N-acetylpenicillamine)-2-amino-2-deoxy-1,3,4,6,-tetra-*O*-acetyl-β-D-glucopyranose

Fig 1. Structural formulas and full generic names for sodium nitroprusside (**A**) and *N*-(*S*-nitroso-*N*-acetylpenicillamine)-2-amino-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-glucopyranose (RIG200; **B**).

stimulate smooth muscle proliferation, alter vasomotor responses, and facilitate infiltration of activated macrophages. Although microthrombi are often associated with such focal erosion, they are believed to be too small to directly cause symptoms. However, more generalized plaque erosion is now recognized as a frequent cause of coronary thrombosis.[14,15] Delivery of exogenous nitric oxide is an attractive therapeutic option to supplement or replace endogenous nitric oxide both in established atherosclerosis and after percutaneous transluminal angioplasty for which unavoidable endothelial damage is a contributory factor in thrombosis and restenosis after the procedure.[16] Indeed, several nitric oxide donors have been shown to prevent the development of thrombosis and hyperplasia in balloon-injured models,[17] and *S*-nitrosoglutathione inhibits platelet activation after coronary balloon angioplasty in humans.[18]

A number of nitric oxide donor drugs, including sodium nitroprusside (Fig 1, *A*) and the organic nitrates, are already in widespread clinical use, but the value of these drugs is limited by several factors. Besides the weak antiplatelet effect of these agents at therapeutic concentrations,[19] prolonged administration of sodium nitroprusside can lead to cyanide poisoning, and organic nitrates engender vascular tolerance.[20,21] Furthermore, current drugs cannot be targeted at areas of endothelial damage and their use can be associated with systemic hypotension.

Recently, *S*-nitrosothiols have been recognized as novel nitric oxide donor drugs that do not appear to engender vascular tolerance.[22,23] We have demonstrated previously that a novel *S*-nitrosated glycoamino acid, *N*-(*S*-nitroso-*N*-acetylpenicillamine)-2-amino-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-glucopyranose (RIG200; Fig 1, *B*), causes prolonged, nitric oxide–mediated vasodilatation in rat femoral arteries only when the endothelium has been removed, suggesting a selective action of RIG200 in vessels with damaged endothelium.[24] These data suggest that RIG200 may be a valuable therapeutic agent but, to date, its clinical pharmacology is unknown.

We tested the hypothesis that RIG200 would cause sustained relaxation in human hand veins with damaged endothelium in vivo and that the effect would not be observed with sodium nitroprusside or with RIG200 in endothelium-intact hand veins. Selectivity for blood vessels with damaged endothelium would support the in vitro data[24] and would indicate the potential of compounds such as RIG200 to protect coronary blood vessels against spasm after coronary balloon angioplasty.

METHODS

Studies were performed to investigate the effect of endothelial damage induced by irrigation with distilled water on the duration of action of brief infusions of sodium nitroprusside and RIG200 (0.06 and 6 nmol/min for both drugs).

Subjects

Fourteen healthy nonsmoking men (age range, 18 to 65 years) were recruited for the study, which was conducted with the approval of the local research ethics committee and with the written informed consent of each subject. All subjects abstained from vasoactive medication in the 2 weeks before the study and from alcohol and caffeine-containing drinks for at least 12 hours before each study. Each subject fasted for at least 3 hours before any measurements were made. All studies were performed in a quiet room maintained at a controlled temperature between 24°C and 26°C.

Drugs

Drugs, with the exception of aspirin, were dissolved in physiologic saline solution (0.9%; Baxter Healthcare

Ltd, Norfolk, UK) supplemented with 1 mg/mL ascorbic acid to prevent oxidation of norepinephrine (Abbott Laboratories Ltd, Kent, England), and infused at locally active concentrations into the hand vein of each subject. The concentrations of sodium nitroprusside (David Bull Laboratories, Warwick, England) and RIG200 were approximately 50% maximum effective concentration (~EC_{50}) and maximal concentrations, as determined in preliminary studies.[25] Acetylcholine was obtained from CIBA Vision Ophthalmics (Southampton, England). All solutions were prepared aseptically from sterile vials or ampules on the day of the study. Three hundred milligrams of aspirin (Approved Pharmaceutical Services Limited, Leeds, England) was administered orally 30 minutes before the start of measurements on the first day of the study and 75 mg aspirin was given on each of the following 2 days to prevent thrombosis in the endothelium-damaged hand vein.[26] RIG200 was synthesized by a published method.[24] Although large-scale toxicologic studies of RIG200 have not yet been performed, data from our preclinical studies[24] and from systemic administration of relatively high doses (2 nmol/kg) to rabbits suggested that the locally active infusions of RIG200 in this study would not have toxic effects. Indeed, no adverse effects were observed with other *S*-nitrosothiols in clinical studies in hand veins,[27] in the forearm circulation,[27,28] in the coronary circulation after balloon angioplasty,[18] or in microvessels in the skin after topical application.[29] Similarly, pilot studies with RIG200 in hand veins did not cause adverse effects.[25]

Intravenous administration

A 23-gauge butterfly needle was sited in a selected dorsal hand vein of the nondominant arm of each subject, in the direction of blood flow without the use of local anesthetic. The same vein was cannulated for each study in that individual. The infusion rate was kept constant throughout at 0.25 mL/min.

Endothelial damage

At the end of the first study visit, the endothelium was damaged by means of a published technique.[25,26,30] In brief, a "withdrawal" needle was placed 3 to 4 cm downstream from the tip of the "infusion" needle, and the segment of the vein was temporarily isolated with use of occluding wedges. For removal of the endothelium, distilled water was perfused through the segment at 5 mL/min for 15 minutes with use of a constant rate infusion-withdraw pump (Harvard Apparatus Inc, South Natick, Mass). The results of a preliminary study (not shown), consistent with earlier work,[25,26,30] indicated that a 15-minute perfusion of distilled water completely abolishes relaxation and causes constriction in response to acetycholine infusion. It has been reported previously that endothelial function remains impaired 2 days after perfusion of distilled water but returns to normal within 14 days.[26]

Measurements

Dorsal hand vein diameter. The response to local intravenous infusion of vasoactive drugs was assessed by measurement of the diameter of the cannulated dorsal hand vein.[31] The nondominant arm was supported above the level of the heart by an arm rest. The diameter of the selected vein, distended by the inflation of an upper arm cuff to 30 mm Hg, was measured by use of a standard displacement technique.[32] In brief, a magnetized lightweight rod rested on the summit of the infused vein ~1 cm downstream from the tip of the infusion cannula. This rod passed through the core of a linear variable differential transformer (model 025 MHR, Lucas Schaevitz Inc, Pennsauken, NJ) supported above the hand by a small tripod, the legs of which rested on areas of the dorsum of the hand free of veins. If venoconstriction occurred while the upper arm cuff was inflated or if the cuff were deflated, with a consequent emptying of the vein, there was a downward displacement of the rod, causing a linear change in the voltage generated by the linear variable differential transformer. The voltage output from the transformer was transferred by a MacLab analog-to-digital converter and Chart software (ADInstruments, Castle Hill, Australia) to a Macintosh personal computer (Apple Computer, Inc, Cupertino, Calif).

Blood pressure. Blood pressure was measured in the noninfused arm with use of a well-validated semiautomated noninvasive method[33] at 30-minute intervals throughout the studies.

Study design

In this double-blind parallel-group study, 14 subjects were randomly assigned to receive either sodium nitroprusside or RIG200 (seven in each group). Each subject was studied on two occasions separated by 2 days. On both study days, saline solution was infused into the hand vein for at least 30 minutes before the administration of drugs to obtain baseline measurements. Increasing doses of norepinephrine (1 to 128 ng/min) were infused sequentially (5 minutes each) until ~70% venoconstriction was achieved. This concentration of norepinephrine (N_{70}) was infused continuously for the remainder of the study. In some volunteers, particularly

Table I. Comparison of baseline parameters between groups before and 2 days after irrigation with distilled water

	*Sodium nitroprusside**		*RIG200†*	
	Before water irrigation	*After water irrigation*	*Before water irrigation*	*After water irrigation*
Norepinephrine dose (ng/min)	6 ± 2	13 ± 5	11 ± 2	37 ± 15
Venoconstriction to norepinephrine (%)	69 ± 5	60 ± 5	69 ± 4	64 ± 7
Reversal of norepinephrine constriction by acetylcholine (%)	27 ± 5	–27 ± 13	30 ± 3	–4 ± 6

Only the response to acetylcholine was significantly affected after irrigation with water in both the sodium nitroprusside ($P = .009$; paired Student t test; n = 7) and RIG200 ($P = .006$) groups. None of the parameters were significantly different between groups ($P > .05$). Negative values indicate a further constriction.

*Age of subjects, 39 ± 5 years.

†Age of subjects, 40 ± 5 years.

on the second visit, venous tone was unstable at N_{70}, and a lower norepinephrine concentration had to be used to ensure a stable baseline. In one volunteer, venoconstriction in response to norepinephrine after the removal of endothelium did not reach 70% constriction within the dose range used in this study. In this case, the highest dose of norepinephrine (128 ng/min; 41% constriction) was used for the study. The endothelium-dependent vasodilator, acetylcholine (1 nmol/min), was then co-infused for 5 minutes and venous responses to acetylcholine were recorded to assess the functional integrity of the endothelium. Acetylcholine was then washed out of the vein and two doses (0.06 and 6 nmol/min) of either RIG200 or sodium nitroprusside were sequentially infused for 10 minutes. The infusion of each dose was followed by a 30-minute washout period. Vein diameter measurements were made at 5-minute intervals throughout the study. Irrigation of the vein with distilled water to cause endothelial damage was performed at the end of the first visit. The subject returned 2 days later, and the same vein was preconstricted and infused with acetylcholine to test endothelial function and then with the same nitric oxide donor drug as that used in the first visit.

Analysis

Baseline vein diameter was calculated as the mean of the last three measurements during the saline infusion, before the start of the active drug infusion. Venodilatation during the infusion of acetylcholine or study drug was expressed as the percentage reversal of N_{70} norepinephrine-induced venoconstriction. Recovery during washout of nitric oxide donor drugs was compared before and after water irrigation in each study group by calculating the area under the curve (AUC). Data were expressed as mean values ± SEM. Unpaired or paired Student t tests were used to analyze differences in mean values between groups (as indicated in the text). A value of $P < .05$ was considered to be statistically significant.

RESULTS

There was no significant difference in the age of subjects or the norepinephrine concentration (N_{70}) between RIG200 and sodium nitroprusside study groups (Table I). There was also no significant change in the blood pressure during the course of the study in subjects receiving either sodium nitroprusside or RIG200, indicating that the effects of the study drugs were confined to the infused arm.

Responses to acetylcholine in intact and water-irrigated hand veins

Before irrigation with water, N_{70}-contracted hand veins of both study groups dilated during perfusion with acetylcholine (Table I). Acetylcholine infusions into the hand veins of subjects 2 days after irrigation with water failed to dilate or even caused further contraction of the veins (Table I). The effect of water irrigation on acetylcholine responses was significant in both study groups ($P = .009$ for sodium nitroprusside and $P = .006$ for RIG200), but there was no significant difference between the effect in the two groups ($P = .17$).

Effect of sodium nitroprusside in hand veins before and after water irrigation

Before irrigation with water, hand veins of subjects in the sodium nitroprusside study group dilated during infusion with low doses (0.06 nmol/min; 78% ± 12%) and high doses (6 nmol/min; 105% ± 11%) of sodium nitroprusside (Fig 2, *A*). Dilatation to both concentrations recovered to around baseline levels (–5% ± 5% and 21% ± 7%, respectively) during the 30-minute washout period.

Similar responses were obtained 2 days after water irrigation, with dilatations of 82% ± 17% and 113% ± 11%

for low and high sodium nitroprusside concentrations, respectively (Fig 2, *A*). Again, dilatation reversed rapidly during washout; the AUC values for the washout periods were not significantly different from those obtained before irrigation ($P = .58$ and $P = .87$, respectively).

Effect of RIG200 in hand veins before and after water irrigation

Before irrigation with water, hand veins of subjects in the RIG200 study group dilated on infusion with low doses (0.06 nmol/min; 59% ± 4%) and high doses (6 nmol/min; 98% ± 5%) of RIG200 (Fig 2, *B*). Dilatation to both concentrations recovered to around baseline levels (–6% ± 10% and 13% ± 11% for low and high concentrations, respectively) during the 30-minute washout period.

Responses of similar amplitude were obtained 2 days after water irrigation, with dilatations of 64% ± 10.2% and 97% ± 7% for the two concentrations, respectively. However, unlike responses to sodium nitroprusside, the responses to RIG200 showed slower recovery on washout, with veins still 48% ± 12% relaxed 30 minutes after washout of the high RIG200 concentration began (Fig 2, *B*). The AUC for the washout period after the low concentration of RIG200 was not significantly different before water irrigation compared with 2 days after water irrigation ($P = .16$; AUC, paired Student *t* test). However, responses to the higher concentration of RIG200 were significantly prolonged after water irrigation ($P = .02$; AUC, paired Student *t* test).

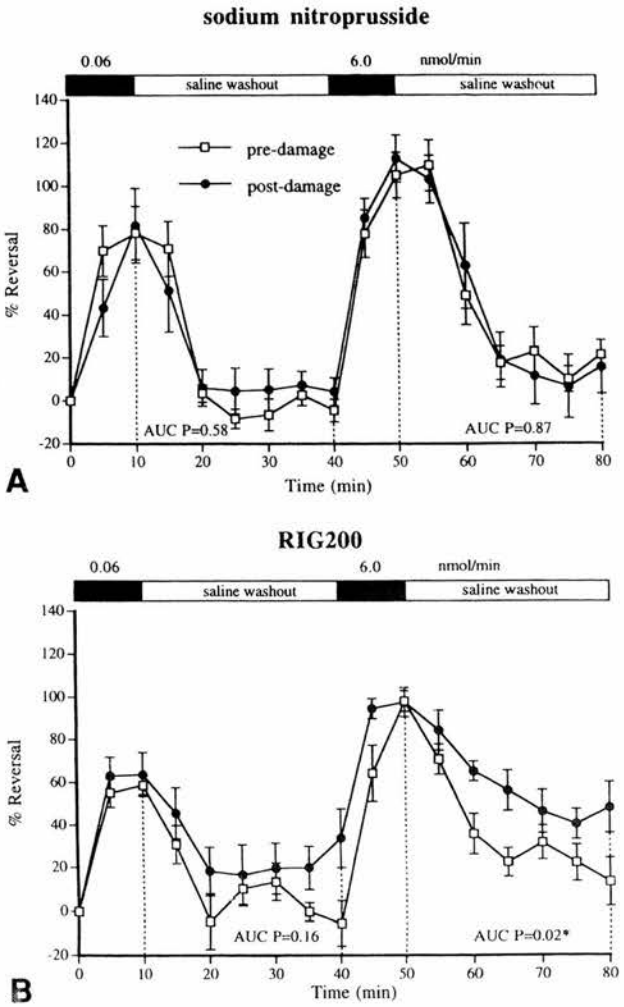


Fig 2. Percentage of reversal of norepinephrine-induced tone by 0.06 and 6.0 nmol/min sodium nitroprusside infusion **(A)** and 0.06 and 6 nmol/min RIG200 infusion **(B)** in hand veins of subjects before and 2 days after 15 minutes of water irrigation to produce endothelial denudation. Negative values indicate further constriction. Area under the curve (AUC) was calculated for responses during washout of each drug concentration. AUC was significantly different after high-dose RIG200 after water irrigation compared with before water irrigation ($P = .02$; paired Student *t* test; n = 7), but not for low-dose RIG200 ($P = .16$) or either of the sodium nitroprusside doses ($P = .58$ and $P = .87$, respectively).

DISCUSSION

Our results show that the novel *S*-nitrosothiol RIG200 is a powerful venodilator in vivo in humans, with a similar efficacy to sodium nitroprusside. Furthermore, we clearly show that responses to RIG200 are prolonged in hand veins that no longer relax to the endothelium-dependent dilator acetylcholine after irrigation with water. This sustained effect is not observed with RIG200 before irrigation with water or with sodium nitroprusside, either before or after water irrigation. The results support earlier findings in rat arteries in vitro[24] and suggest that RIG200 may offer a clinical approach to targeted delivery of nitric oxide to blood vessels with damaged endothelium.

Functional endothelial integrity was assessed in the dorsal hand vein of each subject before and 2 days after irrigation with distilled water with use of the endothelium-dependent dilator acetylcholine. Endothelium-dependent venodilatation was inhibited in both study groups. Indeed, consistent with previous data,[26] acetylcholine caused vasoconstriction in several subjects. It is likely that this endothelial dysfunction is a reflection of cellular damage, and possibly endothelial denudation, induced by irrigation with water. In contrast to earlier reports,[26] hand veins in this study were not more sensitive to norepinephrine after water irrigation. Endothelial damage alone would be expected to increase sensitivity to norepinephrine attributable to abolition of basal nitric oxide generation.[34] However,

our results suggest either that nitric oxide generation in hand veins after stimulation with norepinephrine is relatively low, as suggested previously,[35] or that hypersensitivity of hand veins caused by endothelial damage is compensated for by some smooth muscle damage.

Neither the amplitude nor the duration of responses to sodium nitroprusside was affected by endothelial denudation. In the absence of endothelium-derived nitric oxide, it might be expected that responses to sodium nitroprusside would have been accentuated after endothelial denudation. However, given that basal nitric oxide production in veins is regarded to be low,[35] supersensitivity to exogenous nitric oxide might not be as pronounced as it is in arteries. The amplitude of responses to RIG200 was also unaffected by endothelial denudation, but responses to 6 nmol/min RIG200 were significantly more sustained in veins after irrigation than before. The effect lasted for at least 30 minutes, at which time veins were still ~50% relaxed. Subjects are generally unable to tolerate studies for more than ~3 hours, limiting the washout period we were able to study and preventing determination of the durability of sustained RIG200-induced dilatation of veins with damaged endothelium. However, in vitro studies in rat femoral arteries[24] and human internal mammary arteries have indicated that the effect lasts >4 hours; similar studies in human saphenous veins show sustained activity for ~2 hours.[36]

The mechanism by which RIG200 induces sustained venodilatation is unclear. Our in vitro data show that sustained responses to much higher (1 mmol/L) concentrations of RIG200 are reversed by the nitric oxide scavenger hemoglobin.[24] This result indicates that the sustained action is nitric oxide–mediated and is unlikely to be caused by any cytotoxic effect of RIG200 or nitric oxide. However, the persistence of nitric oxide derived from RIG200 during infusion is highly unlikely in view of the short half-life of nitric oxide in biological environments (3 to 30 seconds). Further in vitro studies have shown that this property of sustained activity is shared by other lipid-soluble *S*-nitrosothiols but not by water soluble compounds.[37] We therefore suggest that damage to the endothelium exposes lipophilic regions in the subendothelial layers that retain RIG200 and other compounds with similar chemical characteristics. Slow release of nitric oxide from the retained compound is sufficient to maintain dilatation after washout of the drug from the lumen.

Drugs such as RIG200 present a promising therapeutic alternative to conventional nitric oxide donors both in established cardiovascular disease and after invasive vascular procedures that cause endothelial damage. In particular, *S*-nitrosothiols with sustained action in areas of endothelial damage may be of benefit in established atherosclerotic plaques in which the endothelium has been eroded, predisposing arteries to microthrombi,[12] and after percutaneous transluminal angioplasty. Our results suggest that RIG200 could prevent local vasospasm immediately after the procedure and, in view of the powerful antiplatelet activity of *S*-nitrosothiols,[18,27] may also help to prevent postprocedural thrombosis. We believe that further experiments are now merited to determine the duration of sustained activity in blood vessels with damaged endothelium and to examine the potential impact of these compounds on plaque stability and restenosis in vessels undergoing angioplasty. In addition, further research is required to determine whether RIG200 is orally active or, like other *S*-nitrosothiols,[29] it can be delivered transdermally.

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PHARMACODYNAMICS AND DRUG ACTION

Big endothelin-3 constricts forearm resistance vessels but not hand veins in humans

Background: **Endothelin-3 (ET-3) and its inactive precursor, big endothelin-3 (big ET-3), are both found in human plasma. We investigated whether big ET-3 is converted to ET-3 in the human forearm resistance vessels and dorsal hand veins in vivo.**

Methods: **In a 4-phase study, 6 subjects received 90 minute intrabrachial artery infusions of big ET-3 (50 and 100 pmol · min^{-1}) and ET-3 (5 and 10 pmol · min^{-1}) in random order. Forearm blood flow was measured by venous occlusion plethysmography. In a second 3-phase study, 6 subjects received 90-minute dorsal hand vein infusions of saline solution, big ET-3 (50 pmol · min^{-1}) and ET-3 (5 pmol · min^{-1}) in random order. In a third 2-phase study, 6 subjects received 90-minute dorsal hand vein infusions of big ET-3 (100 pmol · min^{-1}) and ET-3 (10 pmol · min^{-1}). In the dorsal hand vein studies, vessel diameter was measured by the Aellig technique.**

Results: **Intra-arterial ET-3 caused local forearm vasoconstriction of 20% ± 9% (P = .009) at 5 pmol · min^{-1} and 20% ± 10% (P = .001) at 10 pmol · min^{-1} after 90 minutes, with no difference between doses (P = .69). Intra-arterial big ET-3 also caused local forearm vasoconstriction of 22% ± 6% at 50 pmol · min^{-1} (P = .004) and 18% ± 3% at 100 pmol · min^{-1} (P < .0001) after 90 minutes, with no difference between doses (P = .44). There were no significant differences between the responses to intra-arterial big ET-3 and ET-3 at these doses. Local intravenous ET-3 caused a constriction of 9% ± 2% at 5 pmol · min^{-1} (P = .04) and 22% ± 8% at 10 pmol · min^{-1} (P = .002) after 90 minutes. Big ET-3 at 50 pmol · min^{-1} and 100 pmol · min^{-1} did not affect hand vein tone. All responses were slowly progressive.**

Conclusions: **Based on vasoconstriction, measurable conversion of big ET-3 to ET-3 occurs in forearm resistance vessels but not in dorsal hand veins in vivo. An endothelin-converting enzyme, capable of converting exogenously administered big ET-3 to ET-3, appears to be present in upper limb resistance arteries but not in capacitance vessels in humans. (Clin Pharmacol Ther 2000;68:67-74.)**

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The endothelins (ET-1, ET-2, and ET-3) are a family of 21-amino acid peptides.[1,2] They are extremely potent vasoconstrictor and vasopressor agents, with a characteristically sustained action, and have been shown to contribute to the maintenance of basal vascular tone and blood pressure in humans.[3,4] ET-1 is the predominant isoform in the vascular endothelium[2] and is, therefore, likely to be the isoform of most importance in cardiovascular regulation.

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Two human endothelin receptor subtypes, ET_A and ET_B, have been characterized and cloned.[5,6] The ET_A receptor has a high affinity for ET-1, with an inhibition constant of 0.6 nmol/L^{-1} compared with 140 nmol/L^{-1} for ET-3.[7] In blood vessels, the ET_A receptor is expressed solely on vascular smooth muscle cells where it mediates vasoconstriction.[8] The ET_B receptor has equal affinity for all 3 endothelins, with inhibition constants for ET-1 and ET-3 of 0.12 and 0.06 nmol/L^{-1}, respectively.[7] The ET_B receptor is expressed on endothelial cells,[5] where it mediates vasodilatation,[9] and on vascular smooth muscle cells,[10] where it can contribute to vasoconstriction.[11,12] The balance of responses to endogenous ET-1 at the vascular endothelial and smooth muscle ET_B receptor appears to favor vasodilatation in forearm resistance vessels[13] and in the systemic circulation[14] in vivo in humans.

ET-1 is produced from an inactive 38-amino acid intermediate form, big endothelin-1 (big ET-1), by selective cleavage of the Trp^{21}-Val^{22} bond in the carboxy-terminal of big ET-1 catalyzed by an endothelin-converting enzyme (ECE).[15] In humans, ET-2 and ET-3 are also formed from corresponding intermediates, big ET-2 and big ET-3.[15] Along with ET-1 and big ET-1,[16] immunoreactive big ET-2, ET-3, and big ET-3 have been detected in human plasma[17,18] and preproET-3 mRNA has been detected in blood vessels,[19] suggesting that they might generate and respond to other endothelin isopeptides in addition to ET-1.

Two ECEs have so far been cloned in humans; ECE-1[20] and ECE-2.[21] Both convert big ET-1 in preference to big ET-2 and big ET-3.[21,22] This substrate specificity suggests that there may be yet-undiscovered ECEs selective for big ET-2 or big ET-3. Previous work in our department has demonstrated that exogenously administered big ET-1 constricts human forearm resistance vessels[3] but not human hand veins[23] in vivo. Because circulating blood contains no significant ECE activity[24] and because big endothelins are considered to be inactive without conversion to the mature peptide,[15] these findings suggest that forearm resistance vessels, but not hand veins, contain an ECE able to cleave luminally presented big ET-1 to the mature peptide.

Endogenously generated ET-3 acts mainly at the ET_B receptor, and the balance of actions appears to favor vasodilatation. It would, therefore, be potentially advantageous for a clinically useful ECE inhibitor to inhibit ET-1, but not ET-3 synthesis. However, it is not currently known whether human resistance or capacitance vessels contain an ECE able to generate ET-3 from big ET-3. Therefore, we aimed to determine the existence of such an ECE by infusing big ET-3 into the brachial artery and dorsal hand vein of healthy volunteers and assessing responses by measuring forearm blood flow and dorsal hand vein diameter.

METHODS

Subjects

Eighteen healthy white male subjects between 18 and 60 years of age were recruited to these studies, which were conducted with the approval of the local research ethics committee and with the written informed consent of each subject. All studies were performed in a quiet room, maintained at a constant temperature between 24°C and 26°C. As a further measure of standardization, no subject received vasoactive or nonsteroidal anti-inflammatory drugs in the week before each phase of the study, and all abstained from food for 4 hours, and from alcohol, caffeine-containing drinks and cigarettes for at least 12 hours before any measurements were made.[25]

Drugs

Pharmaceutical grade ET-3 (Peninsula Laboratories, Belmont, Calif) and big ET-3 (Peninsula Laboratories) were administered. A single dose of each peptide was used in individual studies because the slowly progressive and sustained actions of the endothelin isopeptides preclude the use of repeated doses in a single study to examine conventional dose relationships.[26] The peptides were dissolved in physiologic saline solution (0.9%; Baxter Healthcare Ltd, Thetford, UK).

Local intra-arterial administration. The left brachial artery was cannulated with the patient under local anesthesia (1% lidocaine; Astra Pharmaceuticals Ltd, Kings Langley, Herts, UK) with a 27-standard wire gauge needle (Cooper's Needle Works, Birmingham, UK) attached to a 16-gauge epidural catheter (Portex Ltd, Hythe, Kent, UK). Patency was maintained by infusion of saline solution through a Welmed P1000 pump (Welmed Clinical Care Systems, Bramley, Hampshire, UK). The total rate of intra-arterial infusion was maintained constant at 1 mL · min^{-1}.

Local intravenous administration. A 23-gauge butterfly needle (Abbott, Sligo, Republic of Ireland) attached to a 16-gauge epidural catheter was sited in a selected dorsal hand vein, without the use of local anesthesia, in the direction of flow. Patency was maintained by infusion of saline solution through a Welmed P1000 syringe pump. The total rate of intravenous infusion was maintained constant at 0.25 mL · min^{-1}.

Measurements

Forearm blood flow. Blood flow was measured in both forearms by venous occlusion plethysmography[25] by using mercury-in-silicone elastomer strain gauges[27] that

were securely applied to the widest part of each forearm. The hands were excluded from the circulation during each measurement period by inflation of a wrist cuff to 220 mm Hg. Upper arm cuffs were intermittently inflated to 40 mm Hg for 10 seconds out of every 15 seconds to temporarily prevent venous outflow from the forearm and thus allow us to obtain plethysmographic recordings. Recordings of forearm blood flow were made repeatedly over 3-minute periods. Voltage output from 2 single-channel Hokanson EC 4 strain-gauge plethysmographs (DE Hokanson Inc, Bellevue, Wash) was transferred to a Macintosh personal computer (LCIII, Apple Computer Inc, Cupertino, Calif) with a MacLab analogue-to-digital converter and Chart software (v3.2.8: both from AD Instruments Ltd, Castle Hill, Australia). Calibration was achieved by use of the internal standard of the plethysmography unit.

Dorsal hand vein diameter. The left hand was supported above the level of the heart by means of an arm rest. The internal diameter of the dorsal hand vein, distended by inflation of an upper arm cuff to 30 mm Hg, was measured by the Aellig technique.[28] In brief, a magnetized lightweight rod rested on the summit of the infused vein approximately 1 cm downstream from the tip of the infusion cannula. This rod passed through the core of a linear variable differential transformer (LVDT; model 025 MHR, Lucas Schaevitz Inc, Pennsauken, NJ) supported above the hand by a small tripod, the legs of which rested on areas of the dorsum of the hand free of veins. If venoconstriction occurred while this cuff was inflated, or if the cuff was deflated with consequent emptying of the vein, there was a downward displacement of the lightweight rod that caused a linear change in the voltage generated by the LVDT. The voltage output from the LVDT was transferred to a Macintosh personal computer by use of a MacLab analogue-to-digital converter and Chart software. Standard displacements were used to calibrate the LVDT to determine the internal diameter of the vein.

Blood pressure. A well-validated semi-automated noninvasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan) was used to make duplicate measurements of blood pressure in the noninfused arm.[29]

Study design

Forearm blood flow protocol. Subjects were studied on 4 separate occasions and rested recumbent throughout each study. Strain gauges and upper arm cuffs were applied, and the left brachial cannula was sited. Saline solution was infused for 30 minutes, during which 3 measurements of forearm blood flow were made after 5, 15, and 25 minutes, the last measurement being taken as the baseline value. Blood pressure was measured immediately after each forearm blood flow measurement, thereby avoiding any effect on forearm blood flow measurements of the venous congestion caused by this procedure.[30] In random order, and on 4 occasions separated by at least 7 days, subjects received 90-minute brachial artery infusions of ET-3 at 5 and 10 pmol · min^{-1} and big ET-3 at 50 and 100 pmol · min^{-1}. The lower dose of ET-3 was chosen based on previous work showing in vivo that 5 pmol · min^{-1} of ET-3 causes slow-onset vasoconstriction in human forearm resistance vessels, reducing blood flow by approximately 20%.[12] The higher dose of ET-3 (10 pmol · min^{-1}) was chosen with the intention of causing a greater forearm vasoconstriction and reducing blood flow to a similar extent to ET-1 (approximately 40%).[3,26] The doses of big ET-3 were chosen with the intention of producing a measurable forearm vasoconstriction even with only 5% to 10% conversion to ET-3. Although no in vivo studies in human resistance vessels with big ET-3 have been reported, previous work with big ET-1 has shown that 50 pmol · min^{-1} of big ET-1 produces a vasoconstriction comparable to 5 pmol · min^{-1} of ET-1.[3] Forearm blood flow was measured at 5-minute intervals during the infusion of the study agents. Blood pressure was measured at 30, 60, and 90 minutes after the start of the infusion.

Dorsal hand vein protocol. In the first set of studies, subjects were studied on 3 separate occasions separated by at least 7 days, in random order. Subjects rested semirecumbent throughout each study. The dorsal hand vein cannula and the LVDT were sited. Saline solution was infused for 30 minutes, during which vein diameter was measured every 5 minutes. Then, on 3 separate occasions, saline solution, ET-3, or big ET-3 was infused for 90 minutes, with measurement of vein diameter every 5 minutes. The choice of dose of ET-3 was based on previous work showing, in vivo, that 5 pmol · min^{-1} ET-3 causes slow-onset venoconstriction of approximately 5% to 10%.[31] As in the forearm blood flow protocol, the choice of dose of big ET-3 was based on previous work with big ET-1.[23]

Based on the responses obtained to the intravenous infusion of ET-3 (5 pmol · min^{-1}) and big ET-3 (50 pmol · min^{-1}), a further 6 subjects were studied on 2 separate occasions separated by at least 7 days, in random order, by using a higher dose of both ET-3 (10 pmol · min^{-1}) and big ET-3 (100 pmol · min^{-1}).

Data analysis and statistics

Plethysmographic data listings were extracted from the Chart data files, and forearm blood flows were cal-

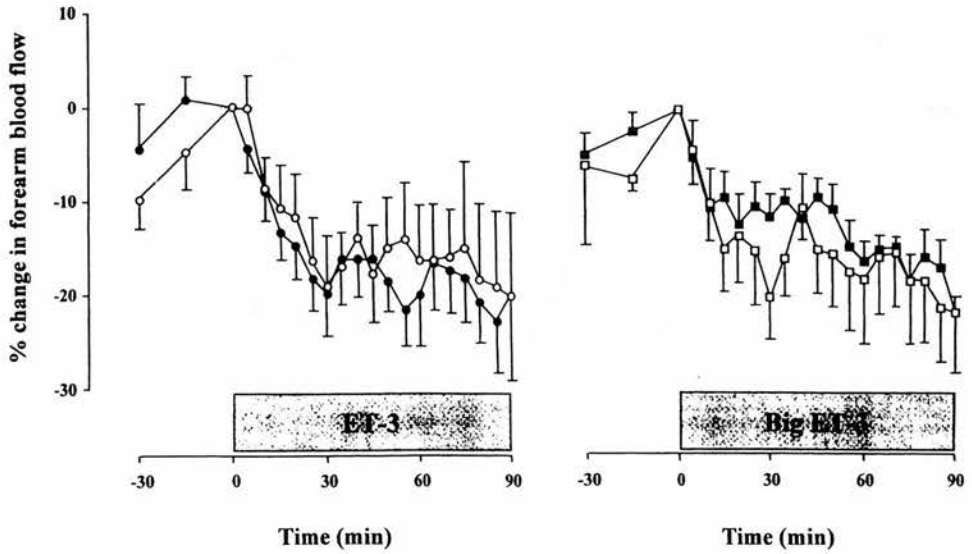

Fig 1. Changes in forearm blood flow after infusion of ET-3 (*open circles*, 5 pmol · min^{-1}; *solid circles*, 10 pmol · min^{-1}) and big ET-3 (*open squares*, 50 pmol · min^{-1}; *solid squares*, 100 pmol · min^{-1}). Significant vasoconstriction occurred after infusion of both peptides at both doses.

Table I. Mean arterial pressure and heart rate before and after 90-minute infusion of sodium chloride, big ET-3, and ET-3

Parameter	*Time*	*Saline solution*	*Big ET-3 (50 pmol · min^{-1})*	*Big ET-3 (100 pmol · min^{-1})*	*ET-3 (5 pmol · min^{-1})*	*ET-3 (10 pmol · min^{-1})*
Intra-arterial protocol						
MAP (mm Hg)	Basal	—	85 ± 3	86 ± 4	84 ± 2	87 ± 4
	90 min	—	86 ± 3	90 ± 3	89 ± 3	91 ± 3
Heart rate (beats/min)	Basal	—	60 ± 3	63 ± 5	59 ± 4	60 ± 5
	90 min	—	59 ± 4	58 ± 5	58 ± 3	61 ± 4
Infused FBF	Basal	—	4.8 ± 1.1	4.1 ± 1.2	4.3 ± 0.8	5.0 ± 1.5
(mL · 100 mL · min^{-1})	90 min	—	4.1 ± 2.0	3.5 ± 1.6	3.6 ± 1.2	4.4 ± 1.5
Intravenous protocol						
MAP (mm Hg)	Basal	85 ± 4	86 ± 3	95 ± 3	85 ± 3	94 ± 2
	90 min	88 ± 3	88 ± 4	98 ± 3	88 ± 5	96 ± 3
Heart rate (beats/min)	Basal	64 ± 5	63 ± 4	72 ± 4	63 ± 4	68 ± 3
	90 min	63 ± 5	65 ± 6	68 ± 3	65 ± 5	69 ± 3
Hand vein size (mm)	Basal	2.4 ± 0.4	2.2 ± 0.2	2.7 ± 0.3	2.2 ± 0.3	2.6 ± 0.3
	90 min	2.5 ± 0.5	2.1 ± 0.2	2.4 ± 0.3	1.9 ± 0.4	2.0 ± 0.4

MAP, Mean arterial pressure; big ET-3, big endothelin-3; ET-3, endothelin-3; FBF, forearm blood flow.

culated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel 4.0; Microsoft Corp, Redmond, Wash). Because wrist cuff inflation results in a transient alteration of forearm blood flow,[32] recordings made in the first 60 seconds after wrist cuff inflation were not used for analysis. Usually, the last 5 flow recordings in each 3-minute measurement period were calculated and averaged for the infused and noninfused arms. To reduce the variability of blood flow data, the ratio of flows in the 2 arms was calculated for each timepoint, in effect by using the noninfused arm as a contemporaneous control for the infused arm.[25] Forearm blood flow results are shown as a percentage change from basal in the ratio of blood flow between infused and noninfused arms.[25]

Basal vein diameter was calculated as the mean of the last 3 measurements before the start of the study infusion, expressed in millimeters.[28] All 3 baseline measurements had to be within 5% of each other before the study infusion was started. To further reduce the already small temporal variability in baseline, the mean of these 3 measurements was used as the baseline value

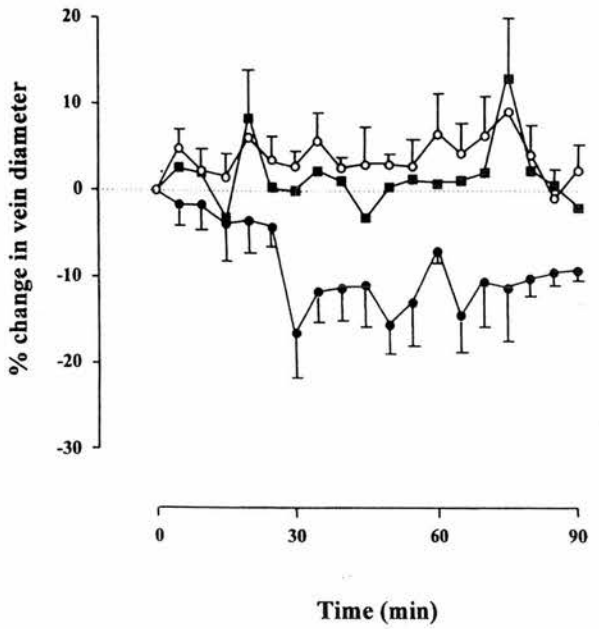

Fig 2. Changes in hand vein diameter after inusion of sodium chloride (*open circles*, 0.9% wt/vol), ET-3 (*solid circles*, 5 pmol · min^{-1}), and big ET-3 (*solid squares*, 50 pmol · min^{-1}). Significant venoconstriction occurred only during infusion of ET-3 ($P = .04$).

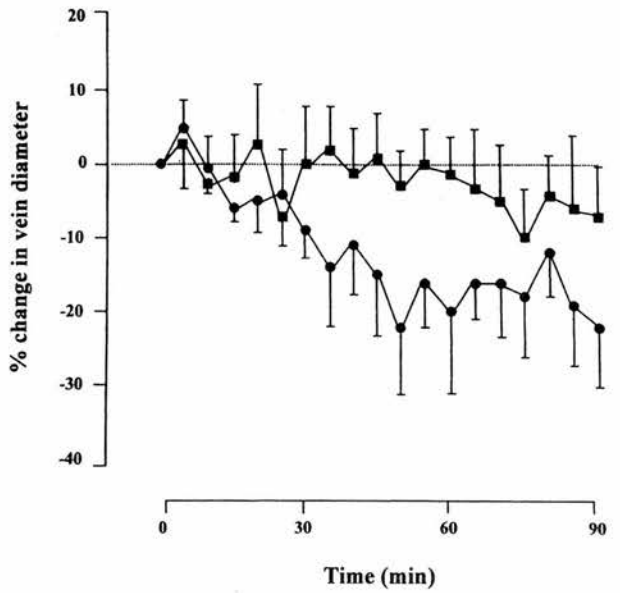

Fig 3. Changes in hand vein diameter after infusion of ET-3 (*circles*, 10 pmol · min^{-1}) and big ET-3 (*squares*, 100 pmol · min^{-1}). Significant venoconstriction occurred only during infusion of ET-3 ($P = .002$).

for calculations. Because basal vein size varies between subjects, responses are expressed as a percentage change in vein size from basal in order to reduce the intersubject variability.[25]

From previous studies that used intrabrachial ET-3 infusions,[12] studying 6 subjects would have an 80% power to detect a 10% vasoconstriction and 95% power to detect a 20% vasoconstriction produced by ET-3 at the 5% level. Our group has not previously infused big ET-3 intra-arterially, but from previous work with intra-arterial big ET-1[3] it was estimated that studying 6 subjects would have a 95% power to detect a vasoconstriction of 20%. Similarly, although our group has no experience with intravenous ET-3 or big ET-3, from previous work with dorsal hand vein infusion of ET-1[33] and big ET-1,[23] it was estimated that studying 6 subjects would have a 90% power to detect a 10% venoconstriction produced by each agent at the 5% level. All power calculations were performed by using GraphPad Instat software (GraphPad Software, San Diego, Calif).

Data are shown as mean values, with 95% CI shown in the text and SEM in the figures. Data were examined by a repeated measures ANOVA with statistical testing of overall significance by Scheffé F test (ANOVA) with Statview 512+ software (Brainpower Inc, Calabasas, Calif) for the Apple Macintosh personal computer.

RESULTS

A total of 14 subjects completed the study, age range 22 to 60 years (mean age, 37 years). Six subjects were included in the forearm blood flow protocol, 12 in the dorsal hand vein protocol, and 4 subjects participated in both the forearm blood flow and the dorsal hand vein protocols. None of the subjects were overweight (mean body mass index, 19) and only 3 were regular smokers.

Basal blood pressure, heart rate, forearm blood flow, and vein diameter were similar on the different study days (Table I). Blood pressure, heart rate, and blood flow in the noninfused arm did not change significantly after infusion of any study agent, confirming that drug effects were confined to the infused arm.

Forearm blood flow protocol

ET-3 at 5 pmol · min^{-1} caused a significant forearm vasoconstriction, with a 20% ± 9% reduction in forearm blood flow at 90 minutes (CI, –1% to –40%; $P = .009$ versus baseline; Fig 1). ET-3 at 10 pmol · min^{-1} also reduced forearm blood flow by 20% ± 10% after 90 minutes (CI, –8% to –33%; $P = .001$ versus baseline; Fig 1). There was no difference in the vasoconstrictor responses between doses ($P = .69$).

Big ET-3 at 50 pmol · min^{-1} caused forearm vasoconstriction, with a 22% ± 6% reduction in forearm

blood flow at 90 minutes (CI, –5% to –38%; $P = .009$ versus baseline; Fig 1). Big ET-3 at 100 pmol · min^{-1} also reduced forearm blood flow by 18% ± 3% after 90 minutes (CI, –8% to –29%; $P = .001$ versus baseline; Fig 1). There were no significant differences between the vasoconstriction produced by the 2 doses of big ET-3 ($P = .44$) or between the vasoconstrictor responses to big ET-3 and ET-3.

Dorsal hand vein protocol

There was no significant change in vein diameter with the placebo treatment, with a change from basal after infusion of saline solution for 90 minutes of +2% ± 3% (CI, –6% to +10%; $P = .66$; Fig 2). Intravenous infusion of big ET-3, at a rate of 50 pmol · min^{-1}, did not affect vein size, with a change at 90 minutes of –2% ± 2% (CI, –12% to +8%; $P = .43$; Fig 2). In contrast, a 10-fold lower dose of ET-3 (5 pmol · min^{-1}) caused progressive venoconstriction of 9% ± 2% at 90 minutes (CI, –6 to –12%; $P = .04$), which was significantly different from the response to saline solution ($P = .001$) and big ET-3 ($P = .008$).

The higher dose of big ET-3 (100 pmol · min^{-1}) also did not affect vein size, with a change at 90 minutes of –7% ± 6% (CI, –22% to +9%; $P = .83$; Fig 3). In contrast, a 10-fold lower dose of ET-3 (10 pmol · min^{-1}) caused a significant venoconstriction of –22% ± 10% at 90 minutes (CI, –46% to –3%; $P = .002$; Fig 3). The difference between the venous responses to these higher doses of ET-3 and big ET-3 was significant ($P = .03$).

DISCUSSION

In these clinical studies, we have shown that both ET-3 and its precursor big ET-3 cause forearm vasoconstriction. We have also shown that ET-3 constricts human hand veins, whereas big ET-3 does not. These findings have important implications for the existence and distribution of an ECE capable of converting big ET-3 to the mature peptide and are consistent with previous findings obtained for big ET-1.

We have shown that intra-arterial infusion of ET-3 causes vasoconstriction of forearm resistance vessels, suggesting the presence of constrictor ET_B receptors and confirming the results of an earlier report.[12] However, the vasoconstriction to ET-3 (5 pmol · min^{-1}) was considerably less than that seen with the nonselective ET_A and ET_B agonist ET-1 (5 pmol · min^{-1}).[3,4,27] This held true even when the concentration of ET-3 infused was increased to 10 pmol · min^{-1}. However, it is difficult to extrapolate these results to quantify the relative contribution of each receptor subtype in mediating the vasoconstrictor effects of endogenous endothelin. Further comparative studies with selective ET_A and ET_B antagonists may help clarify this issue.

Recent reports by our group have provided evidence suggesting that the balance of actions of endogenously generated endothelin acting on ET_B receptors favors vasodilatation.[13,14] These observations may appear to be at odds with the findings presented here. However, in this study the infusions of ET-3 and big ET-3 would have produced much higher local concentrations of ET-3 than normally found physiologically and may either have had direct actions on the vasoconstrictor ET_A receptors or have shifted the balance of ET_B receptor activation away from vasodilatation and toward vasoconstriction. Furthermore, given the role for the ET_B receptor as a clearance receptor,[34] the vasoconstrictor actions of ET-3 may be mediated by ET-1 displaced from ET_B receptors onto unoccupied ET_A receptors. These possibilities could be further explored by coinfusing ET-3 with selective ET_A and ET_B receptor antagonists.

It is highly unlikely that big ET-3 had a direct vasoconstrictor action given its very low affinity for endothelin receptors.[15] Therefore, the vasoconstriction caused by big ET-3 is likely to have resulted from conversion to ET-3. Our studies are consistent with the existence of an ECE capable of converting big ET-3 to ET-3. As both ECE-1 and ECE-2 convert big ET-1 in preference to big ET-3,[35] our study also suggests the possible existence of another ECE in forearm resistance arteries. The slow onset and sustained actions of the endothelin isopeptides precluded us from using repeated doses in a single study to examine conventional dose-relationships. Therefore, the proportion of exogenously administered big ET-3 converted to ET-3 cannot be determined from this study.

It is unlikely that any significant ECE activity is present in human hand veins, because we found no venoconstriction to high doses of big ET-3. However, it is possible that an ECE may be present in these vessels, but not in a location capable of converting exogenous (intraluminal) big ET-3. Hand vein size did not alter during saline solution infusion, confirming that vein size in these experiments is not influenced by repeated measurement or diurnal variation.

The lack of effect of big ET-3 in human hand veins contrasts with the forearm vasoconstrictor actions of the same dose of big ET-3 administered through the brachial artery. Although a greater concentration of infused big ET-3 will have been achieved in the hand veins, it could be argued that infused big ET-3 is in contact with the hand vein under study (approximately 1 cm in length) for insufficient time to allow conversion of big ET-3. However, the concentration of big ET-3

achieved in the vein is sufficient to allow conversion to the mature peptide, given the normal rates of conversion.[15] Similar studies have shown that angiotensin I, infused locally, constricts human hand veins in an angiotensin converting enzyme-dependent manner.[36,37] In contrast with the findings for ET-3 and big ET-3, there is only a small difference in constrictor potency between angiotensin I and angiotensin II in hand veins, similar to the observations with angiotensin peptides in the forearm resistance bed.[36] The lack of conversion of big ET-3 is consistent with the lack of response to big ET-1 in human hand veins[23] despite forearm vasoconstrictor actions of the same dose of big ET-1.[3] Furthermore, given the differences in flow between the forearm circulation and the dorsal hand vein circulation,[25] it is likely that substantially higher concentrations of big ET-1 and big ET-3 are reached in the dorsal hand vein. Thus ECE activity for big ET-1 appears to have a similar vessel distribution to ECE activity for big ET-3.

The endothelin system has been implicated in the pathophysiology of several cardiovascular diseases.[38] Although the major emphasis to date has been in the clinical development of endothelin receptor antagonists, the search for a pathophysiologically relevant ECE and potent ECE inhibitors to prevent endothelin production continues.[39,40] There is a current controversy as to whether combined ET_A and ET_B receptor antagonists are preferable to selective ET_A antagonists, which leave vasodilator ET_B receptors unblocked.[38,41] Indeed, recent reports by our group show that activation of the ET_B receptor by endogenously produced endothelin peptides produces vasodilatation, at least in healthy subjects.[13,14] ECE inhibitors, by blocking generation of ET-1, will act as functional antagonists at both ET_A and ET_B receptors, and so may be less effective vasodilators, under some circumstances, than selective ET_A antagonists. Alternatively, given that ET-1 is the major cardiovascular isoform and ET-3 is a relatively selective agonist at ET_B receptors, ET-1 selective ECE inhibitors could be potentially useful therapeutic agents. The identification and further characterization of the ECE responsible for big ET-1 conversion in vivo may, therefore, be clinically important.

In conclusion, our results show that human forearm resistance vessels have the capacity to convert exogenous big ET-3 to ET-3. In contrast, human hand veins appear to lack this capacity, consistent with our earlier observations with big ET-1. These findings suggest that hand veins exhibit little or no functional ECE activity.

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Constriction to ETB receptor agonists, BQ-3020 and sarafotoxin S6c, in human resistance and capacitance vessels *in vivo*

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Aims The aim of the study was to examine the effects of the ETB receptor selective agonists sarafotoxin S6c (SFTX6c) and BQ-3020 on the forearm resistance and capacitance vessels in healthy subjects *in vivo*.

Methods The local response to intra-arterial or intravenous infusion of SFTX6c (5 pmol min^{-1}) or BQ-3020 (50 pmol min^{-1}) was assessed, on separate occasions, in eight healthy men (aged 20–28 years). Data (mean ± s.e.mean) were examined by ANOVA. Results are expressed as percentage change from baseline at 90 min.

Results SFTX6c and BQ-3020 reduced forearm blood flow, following local intra-arterial infusion (−25 ± 7% and −27 ± 7%, respectively; $P<0.001$) and reduced hand vein diameter, following local intravenous infusion (−30 ± 8% and −16 ± 7%, respectively; $P<0.001$).

Conclusions We have shown that locally active infusions of the selective ETB receptor agonists SFTX6c and BQ-3020 cause arterial constriction and venoconstriction in healthy human blood vessels *in vivo*. These results indicate that ETB receptor stimulation may mediate vasoconstriction in humans.

Keywords: endothelin-1, ETB receptor agonist, vasoconstriction

Introduction

The powerful vasoconstrictor and vasopressor effects of endothelin-1 (ET-1) [1, 2] are mediated via the ET-1 selective, vascular smooth muscle cell ETA receptor [3], while the predominant effect of the nonisopeptide selective ETB receptor [4] would appear to be vasodilatation [5–7] mediated by the endothelial cell ETB receptor. However, ETB receptors have also been described on vascular smooth muscle cells [8] and may contribute to vasoconstriction [9]. Indeed, we have previously demonstrated venoconstriction and vasoconstriction in response to locally active doses of the ETB receptor selective agonist sarafotoxin S6c (SFTX6c) [10] in healthy subjects [11–13] and in patients with heart failure [14].

To address whether the vasoconstrictor effects of SFTX6c [11–14] could also be demonstrated with another, structurally distinct, ETB receptor agonist, we investigated the response to infusion of locally active doses of SFTX6c and BQ-3020, a selective ETB agonist [15, 16], in the forearm resistance and capacitance vessels of healthy subjects *in vivo*. BQ-3020 is a linear analogue of ET-1 with closer structural similarity to ET-1 than SFTX6c [15].

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Methods

Subjects and protocol

Eight healthy men, within the age range of 18–40 years, were recruited to the study, which was conducted with the approval of the local Research Ethics Committee and with the written informed consent of each subject.

The effects of local intra-arterial and intravenous infusion of BQ-3020 and SFTX6c were investigated in eight healthy men in a single-blind, randomised, four way crossover study. On separate occasions, each separated by at least 1 week, each subject received either intra-arterial infusion or intravenous infusion of SFTX6c or BQ-3020. Subjects rested recumbent throughout each study in a

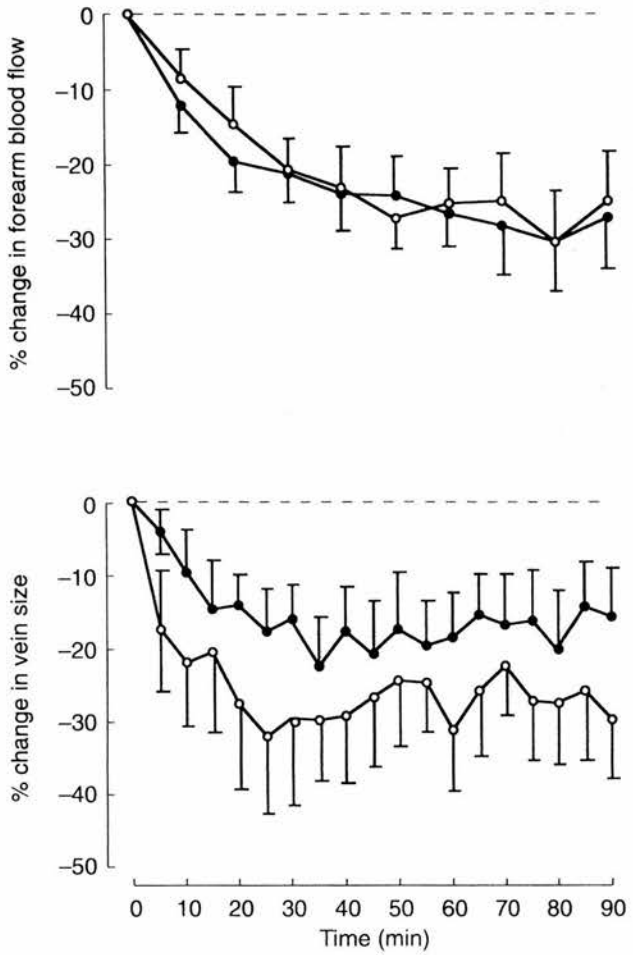

Figure 1 Response of forearm blood flow and hand vein diameter to local intra-arterial infusion and local intravenous infusion of SFTX6c (5 pmol min^{-1}; open circles) and BQ-3020 (50 pmol min^{-1}; closed circles), respectively. Responses are expressed as mean percentage change ± s.e.mean.

quiet temperature-controlled room (23–25°C). Saline (0.9%) was infused for at least 30 min before active drug infusion to allow recording of baseline measurements.

Drugs, administration and measurements

Sarafotoxin S6c and BQ-3020 (both Calbiochem-Novabiochem, Nottingham, UK) were administered by continuous infusion for 90 min. SFTX6c was administered at an infusion rate of 5 pmol min^{-1} as described previously [11–14]. BQ-3020 was administered at an infusion rate of 50 pmol min^{-1}, based on results from a pilot study (data not shown).

The brachial artery of the nondominant arm was cannulated under local anaesthetic (1% lignocaine; Astra Pharmaceuticals, Kings Langley, England) with a 27 SWG steel cannula (Cooper's Needle Works). The infusion rate was kept constant at 1 ml min^{-1} throughout. The response to intra-arterial infusion was assessed by measurement of forearm blood flow using a standard plethysmographic technique [17].

A 23-gauge butterfly needle was sited in a selected dorsal hand vein in the direction of blood flow without the use of local anaesthetic. The same vein was cannulated for each study in that individual. The infusion rate was kept constant throughout at 0.25 ml min^{-1}. The response to intravenous infusion was assessed by measurement of the diameter of the cannulated dorsal hand vein using a standard displacement technique [17, 18].

Blood pressure and heart rate were measured in the noninfused arm using a well-validated semiautomated method [19] at 30 min intervals throughout the infusions.

Statistical analysis

Analysis was performed as described previously [17]. All results are expressed as mean ± standard error of the mean (s.e.mean) at 90 min. Blood pressure, heart rate and baseline measurements were compared using the Student's paired *t*-test. The forearm blood flow and vein diameter responses were examined by repeated-measures analysis of variance (ANOVA) (Excel 5.0, Microsoft Ltd, Wokingham, UK). Statistical significance was accepted at the 5% level.

Results

All subjects, aged 20–28 years, completed the study. There was no significant difference between baseline measurements on each of the study visits. All drug effects were confined to the infused arm [Table 1].

Sarafotoxin S6c and BQ-3020 reduced forearm blood flow ($-25 \pm 7\%$ and $-27 \pm 7\%$, respectively; $P<0.001$), following intra-arterial infusion, indicating vasoconstriction in the infused arm (Figure 1). Both SFTX6c and BQ-3020 caused a reduction in vein diameter following intravenous infusion ($-30 \pm 8\%$ and $-16 \pm 7\%$, respectively; $P<0.001$) (Figure 1).

Discussion

We have confirmed that infusion of a locally active dose of the ETB receptor agonist SFTX6c causes arterial constriction and venoconstriction in healthy human blood vessels *in vivo* [11–13]. We have also extended these observations to show, for the first time, that similar effects are seen with the structurally distinct ETB receptor selective agonist, BQ-3020. These findings support the view that vasoconstriction can occur in response to stimulation of ETB receptors *in vivo*. It has been suggested that this vasoconstriction results from displacement of endogenous ET-1 onto unoccupied ETA receptors [6]. However, this seems unlikely, given that we have shown

Table 1 Mean arterial pressure (MAP), heart rate (HR), forearm blood flow (FBF) and vein diameter at baseline and at 90 min following the start of each infusion. Values are mean ± s.e.mean.

	Intra-arterial infusion		*Intravenous infusion*	
	BQ-3020 (50 pmol min^{-1})	*SFTX6c (5 pmol min^{-1})*	*BQ-3020 (50 pmol min^{-1})*	*SFTX6c (5 pmol min^{-1})*
MAP (mmHg)				
Basal	93±3	86±2	87±2	89±2
90 min	96±5	92±3	86±3	90±3
HR (beats min^{-1})				
Basal	56±3	54±2	57±3	57±3
90 min	56±2	54±3	55±3	58±4
FBF (ml 100 ml^{-1} min^{-1})				
Control arm				
Basal	4.0±0.8	4.4±1.0		
90 min	5.0±1.4	5.2±1.0		
Infused arm				
Basal	4.6±1.0	3.9±0.7		
90 min	3.6±0.7	3.2±0.1		
Vein diameter (arbitrary units)				
Basal			2.7±0.4	2.6±0.3
90 min			2.3±0.3	1.8±0.4

previously that the constrictor effects of SFTX6c could be blocked by the selective ETB receptor antagonist BQ-788 [13] but not by the selective ETA receptor antagonist BQ-123 (unpublished observations).

Although vasoconstriction does appear to occur with ETB receptor selective agonists, the integrated role of endothelial cell dilator and vascular smooth muscle constrictor receptors only emerges from studies with endothelin receptor antagonists. Indeed, we have recently demonstrated local [7] and systemic [5] vasoconstriction in response to the ETB receptor selective antagonist BQ-788 [20], indicating that the balance of effects of endogenous ET-1 acting at vascular smooth muscle and endothelial ETB receptors in healthy resistance vessels favours vasodilatation. Further investigation of the role of the ETB receptor in health and in cardiovascular disease is important to distinguish whether combined ETA/ETB antagonists or selective ETA antagonists will be more effective as vasodilator treatments in the clinical setting.

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Endothelin-1 does not contribute to the release of tissue plasminogen activator in vivo in man

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Summary *Objectives*: Endothelin-1 is a potent endothelium-derived vasoconstrictor peptide with autocrine and paracrine actions. Tissue plasminogen activator (t-PA) and its inhibitor, plasminogen activator inhibitor type 1 (PAI-1), are also released from the vascular endothelium and play a pivotal role in endogenous fibrinolysis. We, therefore, examined the effects of exogenous and endogenous endothelin-1 on t-PA and PAI-1 release in vivo in man.
Design: Open investigative study.
Setting: Clinical Research Centre, University of Edinburgh.
Subjects: Fourteen healthy male volunteers.
Interventions: Unilateral brachial artery infusions of endothelin-1 at 2.5 and 10 pmol/min, and the selective endothelin type B (ET_B) receptor antagonist, BQ-788, at 1 nmol/min.
Main outcome measures: Blood flow and plasma fibrinolytic factors were measured in both forearms using venous occlusion plethysmography and venous blood samples withdrawn from the antecubital fossae.
Results: Endothelin-1 caused a slow onset dose-dependent forearm vasoconstriction ($P<0.001$) with a maximal reduction in blood flow of 40 ± 4% and 63 ± 3% at 2.5 and 10 pmol/min respectively. BQ-788 also caused a slow onset reduction in forearm blood flow ($P<0.001$) reaching a maximum of 21 ± 3%. However, BQ-788 and endothelin-1 did not affect plasma concentrations of t-PA or PAI-1 in the venous effluent of the infused forearm.
Conclusions: Despite sustaining significant vasoconstriction, neither endogenous nor exogenous endothelin-1 influences the release of t-PA or PAI-1 in the forearm vascular bed of man. This suggests that endothelin-1 does not provide a major contribution to the regulation of endogenous fibrinolysis in man. © Harcourt Publishers Ltd 1999

INTRODUCTION

Endothelial cells in the precapillary arterioles and post-capillary venules[1] synthesize and release t-PA and PAI-1 both basally and in response to stimulation by various coagulation factors and stimulants. The time course of t-PA release is important since clot dissolution is much more effective if t-PA is incorporated during clot formation rather than following completion.[2,3] The acute release of t-PA results from the rapid translocation of a dynamic intracellular storage pool[4] and plays a pivotal role in endogenous fibrinolysis.

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Endothelin-1 is a potent endothelium-derived vasoconstrictor peptide with autocrine and paracrine actions. It is continuously released by the endothelium and contributes to the maintenance of basal vascular tone[5] and blood pressure.[6] There are two main endothelin receptor subtypes, ET_A and ET_B, but only the ET_B receptors are present on the endothelium. Endothelin-1 causes vasoconstriction mainly through stimulation of the smooth muscle cell ET_A receptor, although smooth muscle ET_B receptors may also contribute in some vessel types. This vasoconstrictor response is modulated by autocrine endothelial cell ET_B receptor-mediated generation of the endothelium-derived vasodilators, nitric oxide and prostacyclin.

The importance of endogenous t-PA release is exemplified by the high rate of spontaneous reperfusion in the infarct-related artery after acute myocardial infarction, occurring in around 30% of patients within the first 12 h.[7–9]

Following an acute myocardial infarction, plasma endothelin-1 concentrations are elevated and provide an important prognostic marker of survival at 1 year.[10] Furthermore, on the basis of in vitro studies, it has been suggested that endothelin-1 may contribute to the regulation of endogenous fibrinolysis and t-PA release.[11–13] However, the evidence is contradictory, with endothelin-1 being found to either inhibit[13] or stimulate[11,12] endothelial cell t-PA release. The role of endothelin-1 in the regulation of endogenous fibrinolysis in man is currently unknown.

We,[14,15] and others,[16,17] have shown, using bilateral forearm venous occlusion plethysmography and unilateral brachial artery infusions, that the forearm release of t-PA and PAI-1 can be determined in vivo in man. Therefore, the aim of the current study was, using synthetic endothelin-1 peptide and the selective ET_B receptor antagonist, BQ-788, to determine whether endothelin-1, of exogenous or endogenous origin, acts via the endothelial ET_B receptor to regulate the release of t-PA or PAI-1 in vivo in man.

MATERIALS AND METHODS

Subjects

Fourteen healthy men aged between 20 and 33 years participated in three studies which were undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki. The written informed consent of each subject was obtained before entry into the study. None of the subjects received vasoactive or non-steroidal anti-inflammatory drugs in the week before each phase of the study, and all abstained from alcohol for 24 h, and from food, tobacco and caffeine-containing drinks for at least 9 h, before each study. All studies were performed in a quiet, temperature-controlled room maintained at 23.5–24.5°C.

Intra-arterial administration and drugs

The brachial artery of the non-dominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper's Needle Works Ltd, Birmingham, UK) under 1% lignocaine (Xylocaine; Astra Pharmaceuticals Ltd, Kings Langley, UK) local anaesthesia. The cannula was attached to a 16-gauge epidural catheter (Portex Ltd, Hythe, UK) and patency maintained by infusion of saline (0.9%: Baxter Healthcare Ltd, Thetford, UK) via an IVAC P1000 syringe pump (IVAC Ltd, Basingstoke, UK). The total rate of intra-arterial infusions was kept constant throughout all studies at 1 mL/min. Endothelin-1 (Clinalfa AG, Läufelfingen, Switzerland) and BQ-788 (American Peptide Company, Sunnyvale, USA) were administered following dissolution in saline.

Forearm blood flow and blood pressure

Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges applied to the widest part of the forearm.[18,19] During measurement periods the hands were excluded from the circulation by rapid inflation of the wrist cuffs to a pressure of 220 mmHg using E20 Rapid Cuff Inflators (D.E. Hokanson Inc, Washington, USA). Upper arm cuffs were inflated intermittently to 40 mmHg for 10 s in every 15 s to achieve venous occlusion and obtain plethysmographic recordings. Analogue voltage output from an EC-4 Strain Gauge Plethysmograph (D.E. Hokanson) was processed by a MacLab® analogue-to-digital converter and Chart *v*3.3.8 software (AD Instruments Ltd, Castle Hill, Australia) and recorded onto a Macintosh Classic II computer (Apple Computers Inc, Cupertino, USA). Calibration was achieved using the internal standard of the plethysmograph.

Blood pressure was monitored in the non-infused arm at intervals throughout each study using a semi-automated non-invasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc, Tokyo, Japan).[20]

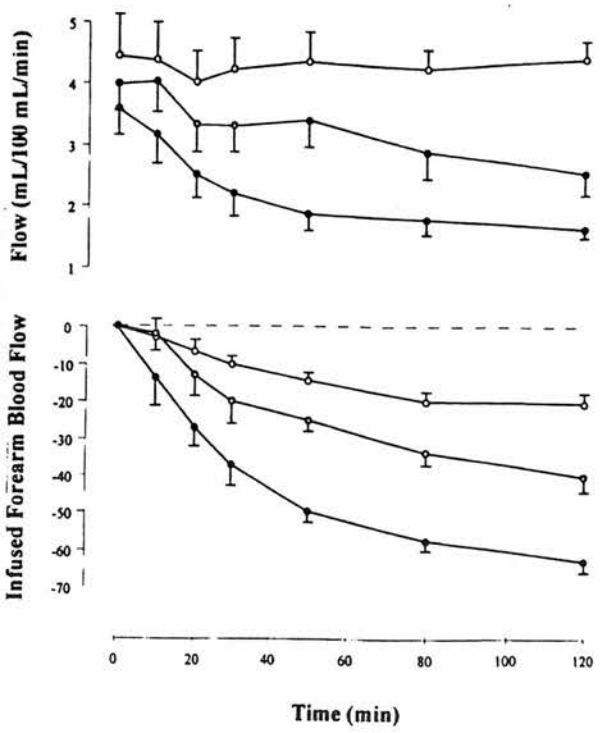
Venous sampling and assays

Venous cannulae (17G) were inserted into large subcutaneous veins of the antecubital fossa in both arms. Ten mL of blood was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool® Stabilyte™, Umeå, Sweden; for t-PA assays) and citrate (Monovette®, Sarstedt, Nümbrecht, Germany; for PAI-1 assays) tubes, and kept on ice before being centrifuged at 2000 g for 30 min at 4°C. Platelet-free plasma was decanted and stored at –80°C before assay.[21]

Plasma PAI-1 and t-PA antigen concentrations were determined using an enzyme-linked immunosorbent assay (ELISA); Coaliza® PAI-1 [22] and Coaliza® t-PA[23] (Chromogenix AB, Mölndal, Sweden) respectively. Plasma t-PA activities were determined by a photometric method, Coaset® t-PA[24] (Chromogenix AB). Intra-assay coefficients of variation were 7 and 5.5% for t-PA and PAI-1 antigen, and 4% for t-PA activity respectively. Inter-assay coefficients of variability were 4, 7.3 and 4% respectively. The sensitivities of the assays were 2.5 ng/mL, 0.5 ng/mL and 0.10 IU/mL respectively. Haematocrit was determined by capillary tube centrifugation of blood anticoagulated by ethylene diamine tetraacetic acid and was obtained from the infused forearm at baseline and at 120 min.

Study design

On each study day, subjects attended fasted and rested recumbent throughout the study. Strain gauges and cuffs



1 Absolute (mL/100 mL of tissue/min: upper panel) and
:entage (% relative to the non-infused forearm: lower panel)
nge of blood flow in the infused forearm during intra-arterial
sion of BQ-788 (1 nmol/min;○) and endothelin-1 (2.5
)l/min:○ and 10 pmol/min: ●).

:e applied and the brachial artery of the non-domi-
nt arm cannulated. Throughout each of the studies,
asurements of forearm blood flow were made every 10
n. Before drug administration, saline was infused for
min to allow time for equilibration and the final blood
v measurement during saline infusion was taken as
basal forearm blood flow.

Eight subjects received an intra-brachial infusion of endothelin-1 at 2.5 and 10 pmol/min for 120 min, given in random order, on two separate occasions, at least 1 week apart. Eight subjects (two had also attended for endothelin-1 infusions) received an intra-brachial infusion of BQ-788 at 1 nmol/min for 120 min. Venous samples were withdrawn from each arm at baseline and at 10, 20, 30, 50, 80 and 120 min after the start of endothelin-1 or BQ-788 infusion.

Data analysis and statistics

Plethysmographic data were extracted from the Chart data files and forearm blood flows were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel *v5*; Microsoft Corporation, Cambridge, USA). Recordings from the first 60 s after wrist cuff inflation were not used because of the reflex vasoconstriction this causes.[18,19] Usually, the last five flow recordings in each 3 min measurement period were calculated and averaged for each arm. To reduce the variability of blood flow data, the ratio of flows in the two arms was calculated for each time point: in effect using the non-infused arm as a contemporaneous control for the infused arm.[18,19] Percentage changes in the infused forearm blood flow were calculated[18,19] as follows:

$$\% \text{ Change in blood flow} = 100 \times \{I_t/NI_t - I_b/NI_b\} / I_b/NI_b$$

Where I_b and NI_b are the infused and non-infused forearm blood flows at baseline (time 0) respectively, and I_t and NI_t are the infused and non-infused forearm blood flows at a given time point respectively.

Estimated net release of t-PA activity and antigen was defined previously[14,15] as the product of the infused forearm plasma flow (based on the mean haematocrit, HCt,

le 1 Systemic haemodynamics, forearm blood flow and haematocrit at baseline and after intra-arterial infusion for 120 min.

	BQ-788 1 nmol/min		Endothelin-1 2.5 pmol/min		Endothelin-1 10 pmol/min	
	Basal	Final	Basal	Final	Basal	Final
od pressure (mmHg)						
olic	130 ± 5	134 ± 6	136 ± 3	140 ± 4	133 ± 4	133 ± 4
tolic	75 ± 4	77 ± 4	72 ± 3	71 ± 3	70 ± 3	73 ± 4
rt rate (/min)	59 ± 3	60 ± 3	62 ± 4	58 ± 4	61 ± 5	62 ± 4
olute forearm blood flow (mL/100 mL/min)						
-infused arm	3.1 ± 0.4	3.8 ± 0.2	3.4 ± 0.3	3.7 ± 0.5	3.1 ± 0.3	4.1 ± 0.3
sed arm	4.5 ± 0.7	4.2 ± 0.4*	4.0 ± 0.4	2.5 ± 0.3*	3.6 ± 0.4	1.6 ± 0.1*
o of infused/non-infused	1.35 ± 0.15	1.14 ± 0.06†	1.13 ± 0.03	0.42 ± 0.03†	1.05 ± 0.07	0.40 ± 0.03†
matocrit	0.41 ± 0.01	0.41 ± 0.01	0.42 ± 0.02	0.40 ± 0.02‡	0.41 ± 0.01	0.40 ± 0.01‡

0.001 (two-way ANOVA; infused vs non-infused)
0.001 (one-way ANOVA)
0.05 (paired t-test; basal vs final)

and the infused forearm blood flow, FBF) and the concentration difference between the infused ($[t\text{-}PA]_{Inf}$) and non-infused arms ($[t\text{-}PA]_{Non\text{-}inf}$).

$$\text{Estimated net t-PA release} = FBF \times \{1\text{-}HCt\} \times \{[t\text{-}PA]_{Inf} - [t\text{-}PA]_{Non\text{-}inf}\}$$

Data were examined by two way analysis of variance (ANOVA) with repeated measures and two-tailed paired Student's t-test using Excel *v*5.0 (Microsoft) where appropriate. All results are expressed as mean ± SEM. Statistical significance was taken at the 5% level. Based on previous data,[14,15] the study had 90% power to detect a 20% change in plasma t-PA concentrations between treatment periods at the 5% level.

RESULTS

All subjects were normotensive and there were no significant changes in blood pressure, heart rate or blood flow in the contralateral arm throughout any of the studies (Table 1). Haematocrit decreased slightly in each endothelin study (Table 1). Between the 3 protocols there were no significant differences in the baseline values of blood pressure, heart rate, forearm blood flow, haematocrit or plasma concentrations of t-PA and PAI-1.

Endothelin-1 infusions

Endothelin-1 decreased blood flow in the infused arm ($P<0.001$) in a dose-dependent manner (Fig. 1) reaching a minimum of 2.5 ± 0.3 mL/100 mL/min at 2.5 pmol/min and 1.6 ± 0.1 mL/100 mL/min at 10 pmol/min, after 120 min. This corresponds to a relative reduction in forearm blood flow of 40 ± 4% and 63 ± 3% respectively. The plasma concentrations of t-PA and PAI-1 did not change in the infused arm (Fig. 2) during endothelin-1 infusion at either concentration (P=NS; one-way ANOVA). In comparison to the non-infused arm, there was a trend (P=0.06; two-way ANOVA) for the infused forearm plasma t-PA antigen concentration to be greater with 10 pmol/min of endothelin-1. However, there were no significant differences in plasma concentrations of PAI-1 antigen (Table 2) or t-PA activity between the forearms.

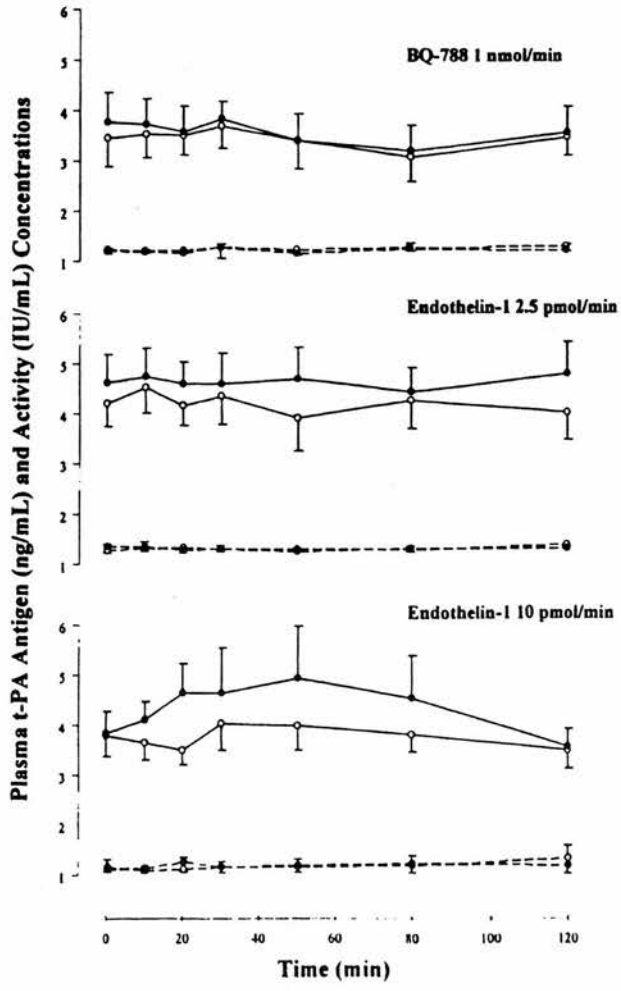


Fig. 2 Plasma concentrations of tissue plasminogen activator (t-PA) antigen (ng/mL; solid lines) and activity (IU/mL; dashed lines) in the infused (solid circles) and non-infused (open circles) forearm during intra-arterial infusion of BQ-788 (1 nmol/min) and endothelin-1 (2.5 and 10 pmol/min).

Table 2 Plasma plasminogen activator inhibitor type 1 (PAI-1) concentrations (ng/mL) during endothelin-1 (ET-1) and BQ-788 infusion. Mean ± SEM.

	Time (min)						
	Baseline	**10**	**20**	**30**	**50**	**80**	**120**
BQ-788 (1 nmol/min)							
infused arm	39 ± 15	37 ± 13	43 ± 15	41 ± 13	37 ± 12	37 ± 12	29 ± 9
non-infused arm	38 ± 13	40 ± 14	43 ± 14	41 ± 13	37 ± 13	33 ± 9	32 ± 9
ET-1 (2.5 pmol/min)							
infused arm	28 ± 7	27 ± 5	26 ± 6	26 ± 5	25 ± 5	21 ± 5	20 ± 5
non-infused arm	27 ± 5	29 ± 6	29 ± 6	27 ± 5	28 ± 7	25 ± 5	23 ± 4
ET-1 (10 pmol/min)							
infused arm	25 ± 5	26 ± 4	27 ± 5	26 ± 5	23 ± 5	21 ± 4	22 ± 5
non-infused arm	27 ± 5	27 ± 5	26 ± 5	25 ± 4	24 ± 5	23 ± 5	22 ± 4

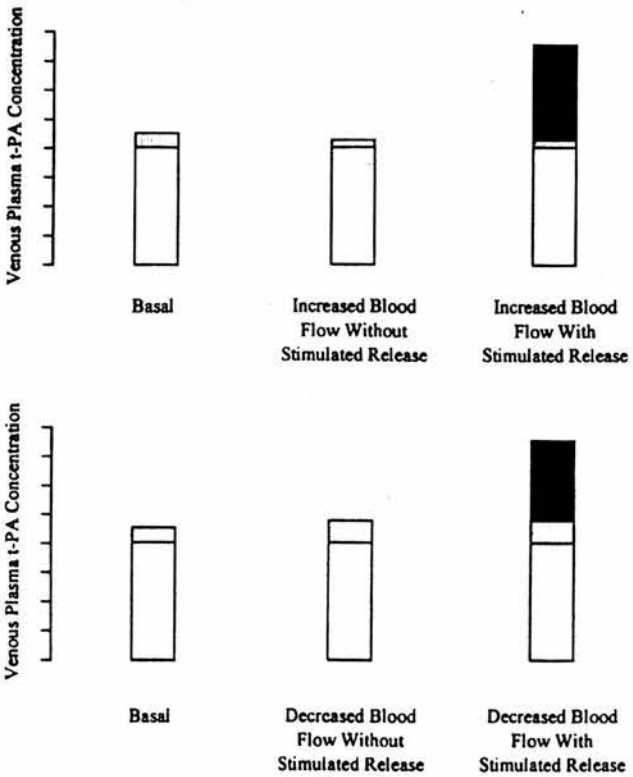


Fig. 3 Theoretical components of venous plasma t-PA concentration under basal conditions and during increases (upper panel) and decreases (lower panel) in blood flow with and without direct stimulation of t-PA release. Open bars: circulating or arterial t-PA; grey bars: basal 'constitutive' t-PA released from the tissue bed; black bars: stimulated 'facultative' t-PA released from the tissue bed

BQ-788 infusion

In comparison to the non-infused arm, BQ-788 decreased blood flow in the infused forearm after 120 min (relative reduction of 21 ± 3%) although the absolute blood flow was unchanged (Fig. 1 and Table 1). The plasma concentrations of t-PA and PAI-1 (Fig. 2 and Table 2) did not change in the infused forearm (P=NS; one-way ANOVA) or in comparison to the non-infused forearm (P=NS; two-way ANOVA).

There was no significant net release of t-PA with infusions of either endothelin-1 or BQ-788 (Table 3).

DISCUSSION

We have demonstrated that, despite causing significant reductions in blood flow, neither endogenous nor exogenous endothelin-1 influences the release of t-PA or PAI-1 in the forearm vascular bed of man. This suggests that endothelin-1 does not contribute to the regulation of endogenous fibrinolysis in man.

Endothelial cell culture techniques have limitations in the investigation of t-PA release and may not be truly representative of the in vivo function of these cells. The amount of t-PA released in culture is small and necessitates prolonged incubation periods and sensitive assays. Moreover, the phenotype of endothelial cells in culture, and the ability to release t-PA, changes with increasing passages. This may account for the disparity of our findings with previous endothelial cell culture studies.[13]

Studies in intact whole animals have suggested that systemic endothelin-1 infusion is associated with stimulation of t-PA release,[12] although plasma t-PA concentrations are not increased by low sub-pressor doses of endothelin-1 in man.[25] Systemic endothelin-1 administration, particularly at pressor doses, will induce changes in cardiac function and regional blood flow as well as having widespread effects on disparate tissues. Thus, the consequent changes in systemic fibrinolytic parameters will be a combination of many factors, potentially including hepatic production and clearance of t-PA and PAI-1. One approach, to avoid these confounding systemic effects, has been to use the isolated perfused rat hindlimb model. This ex vivo model has been reported to demonstrate that endothelin-1 infusion stimulates modest amounts of t-PA release.[11] However, this increased 'release' may, in part, reflect the concentrating effects of a reduction in blood flow associated with endothelin-1 infusion and the concentrations of endothelin-1 administered. In studies conducted to date,[11-13] endothelin-1 has been administered in nanomolar concentrations.

Table 3 Estimated net release of tissue plasminogen activator (t-PA) antigen across the forearm during endothelin-1 (ET-1) and BQ-788 infusion. Mean (95% confidence intervals)

	Time (min)						
	Baseline	10	20	30	50	80	120
t-PA release (ng/100 mL/min)							
BQ-788 (1 nmol/min)	1.1 (–0.1 to 2.3)	0 (–1.6 to 1.6)	0.4 (–1.0 to 1.8)	0.3 (–1.5 to 2.1)	–0.2 (–1.8 to 1.4)	0.1 (–1.3 to 1.5)	0.1 (–1.7 to 1.9)
ET-1 (2.5 pmol/min)	0.1 (–0.7 to 0.9)	1.4 (–0.6 to 3.4)	2.5 (0.5 to 4.5)	1 (–0.8 to 2.6)	2 (–1.0 to 5.0)	1.7 (–1.1 to 4.5)	0 (–0.2 to 0.2)
ET-1 (10 pmol/min)	1.3 (–0.3 to 2.9)	0.5 (–0.4 to 1.9)	0.9 (0.1 to 1.7)	0.3 (–0.4 to 1.7)	0.9 (–0.1 to 1.9)	0.1 (–0.7 to 0.9)	0.8 (0.0 to1.6)

Although local abluminal concentrations may be high, normal human plasma endothelin-1 concentrations are in the femtomolar range. Indeed, in the present study, assuming a total forearm blood flow of 30–50 mL/min, the forearm tissue concentration of endothelin-1 during the 10 pmol/min infusion will be 200–300 fmol/mL. The previous ex vivo animal studies,[11] therefore, represent some 4–5 orders of magnitude higher concentrations and the release of t-PA is likely to represent a pharmacological rather than physiological effect.

We have not detected a significant release of t-PA from the forearm with endothelin-1 infusion despite a 63% reduction in blood flow at the higher dose. Basal t-PA release is of the order of ~0.9 ng/100 mL of tissue/min in the forearm[16] and the apparent trend for an increase in t-PA antigen concentrations may, in part, reflect the reduction in blood flow associated with the marked forearm vasoconstriction (see Fig. 3). This is borne out by the unchanged t-PA activity, because it would be anticipated that plasma PAI-1 and t-PA antigen concentrations would increase proportionately with reductions in blood flow. ET_B receptor antagonism causes both inhibition of endothelium-derived vasodilators such as nitric oxide, and potential hyperstimulation of the unopposed ET_A receptor. However, as with endothelin-1, BQ-788 did not affect plasma concentrations of t-PA or PAI-1 in the infused forearm.

Forearm release of t-PA has been demonstrated using various endothelial cell stimulants including methacholine,[16,26] noradrenaline[17] and desmopressin.[27] Using the same technique as in the present study, we have previously demonstrated in vivo t-PA release of up to 80 ng/100 mL of tissue/min across the human forearm using intrabrachial substance P infusion[14] and this release is sustained for at least 2 h.[15] In contrast, stimulation or antagonism of the endothelial ET_B receptor, with endothelin-1 and BQ-788 respectively, does not appear to influence forearm t-PA release. It is, therefore, unlikely that endothelin-1 provides a major contribution to the regulation of t-PA release in man, although we cannot exclude a small stimulatory effect.

Study limitations

In the forearm, typical resting arterio-venous differences are only ~10% of the total venous t-PA concentration and the basal constitutive release of t-PA antigen is ~0.9 ng/100 mL of tissue/min.[16] We have measured venous–venous differences between the infused and non-infused arms which, unlike the measurement of arterio–venous differences of the infused arm, has the disadvantage of not being able to correct for blood-flow-dependent changes in venous plasma t-PA concentrations. Theoretically (see Fig. 3), in the absence of an alteration in t-PA release, a 60% reduction in blood flow would be anticipated to increase total venous plasma t-PA concentrations by only ~7%, whereas a 200% increase in flow would reduce t-PA concentrations to the same degree (~7%). In the presence of stimulated t-PA release, these small flow-dependent changes are proportionately reduced even further.

The measurement of arterio–venous differences necessitates arterial sampling and the insertion of large-bore cannulae (19–20 gauge) which do not lend themselves to multiple cannulations within the same subject. Moreover, there is also the potential to introduce artefact from the presence of a larger thrombogenic surface, given that activated factor Xa is the most potent stimulant for t-PA release yet known.[28] To minimize arterial trauma and facilitate repeated studies in the same subjects, we have used 27-gauge arterial cannulae which permit drug infusion but not arterial blood sampling. However, we would suggest that flow-dependent changes in venous t-PA concentrations are small, within the variability of the t-PA assays (~5–7%) and are not of practical importance. Interestingly, a significant fall in the arterio–venous difference, or venous plasma concentration, of t-PA has not been detected during blood flow increases of up to 600% with sodium nitroprusside infusion.[14,16,29]

Measurement of venous–venous and arterio–venous differences both have the potential limitation that they can only estimate the net release of t-PA from the forearm and are unable to take account of clearance of t-PA within the forearm. However, the majority of t-PA is removed from the circulation by the liver[30] and the contribution of forearm clearance of t-PA is, therefore, likely to be very small.

During the present study, we did not see changes in heart rate or blood pressure to suggest systemic effects of endothelin-1[31] or BQ-788 infusion.[32] However, measuring venous concentrations bilaterally will control for any potential systemic effects which may go unrecognized if arterio–venous differences are measured in isolation. Once a drug has a systemic rather than a local effect, there is always the concern that subsequent t-PA release may be influenced or mediated by the release of other humoral factors, such as catecholamines. Moreover, if the main mechanism of t-PA release is mediated by a systemically released intermediate factor, then measuring arterio–venous differences could fail to detect this since arterial concentrations may remain unchanged and venous concentrations will rise in both forearms.

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Systemic Blockade of the Endothelin-B Receptor Increases Peripheral Vascular Resistance in Healthy Men

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Abstract—Endothelin-1 (ET-1) is an important mediator of vascular tone in humans, and a number of endothelin receptor antagonists are currently in clinical development as vasodilator agents. While the vasoconstrictor role of the ET_A receptor is undisputed, the role of the ET_B receptor remains unclear. Hemodynamic effects of systemic doses of the ET_B-selective antagonist BQ-788 were investigated in 5 healthy male volunteers (age range, 33 to 48 years) in a placebo-controlled, four-way crossover study. After a 15-minute infusion of BQ-788 (3, 30, or 300 nmol/min) or placebo, plasma ET-1 and big ET-1, blood pressure, heart rate, cardiac index, and stroke index were measured. Total peripheral vascular resistance was calculated from cardiac index and mean arterial pressure. Hemodynamic data are expressed as maximum, placebo-corrected, percentage change from baseline following BQ-788 (300 nmol/min) and were examined by ANOVA. Plasma ET-1 increased by 3.7±1.2 pg/mL (maximum at 15 minutes, $P=0.02$), whereas there was no significant change in plasma big ET-1. Although BQ-788 had no effect on mean arterial pressure, there was a reduction in heart rate (13±7% at 50 minutes; $P=0.002$), cardiac index (17±5% at 40 minutes; $P<0.0001$), and stroke index (8±4% at 40 minutes; $P=0.002$) and an increase in total peripheral vascular resistance (24±5% at 40 minutes; $P<0.0001$). The selective ET_B receptor antagonist BQ-788 causes peripheral vasoconstriction in healthy volunteers, suggesting that the overall balance of effects of endogenous ET-1 at the vascular ET_B receptor favors vasodilatation. Further investigation is now clearly required to address whether selective ET_A or combined ET_A/ET_B receptor blockade will be more effective in the clinical setting. (***Hypertension*. 1999;33[part II]:581-585.)**

Key Words: endothelin ■ vasoconstriction ■ blood pressure ■ receptors, endothelin ■ endothelin receptor antagonist

The importance of endothelin-1 (ET-1) as a mediator of basal vascular tone in vivo in humans has been demonstrated by local[1-3] and systemic[2] vasodilatation in response to endothelin receptor antagonism. The potent vasoconstrictor effects of ET-1,[4,5] combined with the increased plasma concentrations of ET-1 associated with cardiovascular diseases, including heart failure[6] and renal failure,[7] provide strong evidence to support a functional role for ET-1 in the development and maintenance of the increased peripheral vascular resistance associated with these conditions.

The vascular effects of ET-1 are mediated by two distinct receptors: the ET-1–selective ET_A receptor[8] and the nonisopeptide-selective ET_B receptor.[9] The sustained vasoconstrictor effects of ET-1 are predominantly mediated by the ET_A receptor, although vascular smooth muscle ET_B receptors have also been described[10] and may, under some circumstances, contribute to ET-1–mediated vasoconstriction in animal models[11] and humans in vivo.[12] ET_B receptors were first described on endothelial cells, where they act to modulate the vasoconstrictor effects of ET-1 through generation of nitric oxide[13] and prostacyclin.[14] The ET_B receptor also has a role in the clearance of ET-1 from the circulation,[15] although the exact site of the clearance receptor remains to be confirmed. The contribution of the vascular ET_B receptor to the recognized endogenous ET-1–mediated constrictor tone depends on the balance between the ET_B receptor–mediated effects of vasodilatation, vasoconstriction, and ET-1 clearance.

Local vasoconstriction to ET_B receptor agonists has been described in healthy volunteers[12,16] and in patients with heart failure.[17] However, more recently, vasoconstriction after local administration of the selective ET_B receptor antagonist BQ-788[18] has been described in healthy volunteers[3] and in patients with heart failure.[19] The results with antagonists are particularly important as they indicate that the endogenous effect of vascular ET_B receptor stimulation in vivo favors vasodilatation. Indeed, hypertension has been described after administration of systemic doses of the selective ET_B receptor antagonists A192621 in rats and BQ-788 in rabbits in vivo, as well as in rescued ET_B knockout mice.[20,21] The vasoconstrictor effects of ET_B antagonism may result directly from blockade of an endothelial ET_B receptor–mediated dilator

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tone or indirectly from displacement of endogenously generated ET-1 to vasoconstrictor ET_A receptors, or as a result of reduced clearance of ET-1 by vascular ET_B receptors. Confirmation of the balance of the vascular effects mediated by the ET_B receptor in different circumstances is important in understanding the physiology of the endothelin system and in determining whether selective ET_A receptor antagonists or combined ET_A/ET_B receptor antagonists are likely to be more effective vasodilator agents in the clinical setting. Although both selective and nonselective endothelin receptor antagonists have demonstrated vasodilator effects in healthy subjects,[1,2] in patients with heart failure[22,23] and in patients with hypertension,[24,25] the question of whether selective ET_A or combined ET_A/ET_B receptor antagonism will be of more benefit as vasodilator therapy remains to be clarified.

As a first step in understanding the contribution of the ET_B receptor to the maintenance of vascular tone in vivo, we investigated the systemic hemodynamic effects of BQ-788 in healthy male volunteers.

Methods

Subjects

Five healthy male subjects between 18 and 50 years of age were recruited to the study, which was performed in the Clinical Research Center at the Western General Hospital, Edinburgh, with the approval of the local research ethics committee and the written informed consent of each subject. The investigations conformed with the principles outlined in the Declaration of Helsinki. No subject received vasoactive medication or nonsteroidal anti-inflammatory drugs in the week before each phase of a study, and all subjects abstained from alcohol for 24 hours and from food, caffeine-containing drinks, and tobacco for at least 4 hours before any measurements were made. All studies were performed in a quiet room kept at a controlled temperature between 22°C to 24°C.

Drugs

BQ-788 (Clinalfa AG) was used as a selective ET_B receptor antagonist on the basis of both a 1000-fold selectivity of BQ-788 for the ET_B receptor, in the nanomolar range, in human cell lines[18] and inhibition of ET-3 binding to recombinant human ET_B receptors expressed in Chinese hamster ovary cells, also in the nanomolar range.[26] The dose range (3 to 300 nmol/min) used in the current study was selected from previous work investigating the local effects of BQ-788 in the forearm circulation[3] and from a dose ranging pilot study in which 2 volunteers were studied at each dose level (data not shown). Selected doses (1 to 300 nmol/min) were administered in the pilot study to identify a no-effect dose and select an appropriate maximum dose for the main study.

BQ-788 was dissolved in physiological saline (0.9%, Baxter Healthcare, Ltd). Saline (0.9%, Baxter Healthcare, Ltd) was administered as placebo. BQ-788 and placebo were administered in a single-blind manner and infused intravenously at a constant rate for 15 minutes via an 18 standard wire gauge (SWG) cannula sited in the left antecubital vein. All solutions were prepared from sterile stock solutions on the day of the study.

Measurements

Plasma ET-1 and Big ET-1

Blood samples were obtained before dose and at 5, 15, 60, and 240 minutes after dose via an 18 SWG cannula sited in the noninfused arm. In brief, 10-mL samples were collected into sterile EDTA tubes (K3 EDTA, Vacutainer, Becton Dickinson Vacutainer Systems), centrifuged immediately at 2000g for 20 minutes, and stored in plain tubes at −80°C before assay. ET-1 and big ET-1 (Peninsula Laboratories Europe) were determined by standard radioimmunoassay, as previously described.[27,28]

Blood samples were also taken on admission and before discharge for routine biochemistry and hematology blood tests (sodium, potassium, creatinine, urea, alkaline phosphatase, γ-glutamyl transpeptidase, hemoglobin, and white cell count).

Hemodynamic Recordings

Hemodynamic recordings were made at 10-minute intervals from 30 minutes before dose until 1 hour after the start of the infusion, with an additional blood pressure measurement at 15 minutes corresponding with the end of the infusion. Recordings were again made at 30-minute intervals until 2 hours and hourly until 4 hours after the start of the infusion.

Blood pressure and heart rate (HR) were recorded in duplicate at each time point using a semiautomated noninvasive oscillometric method in the noninfused arm (Takeda UA 751 sphygmomanometer, Takeda Medical Inc)[29]; values were averaged for each time point. Blood pressure is presented as mean arterial pressure (MAP; diastolic blood pressure + 1/3 pulse pressure, in millimeters of mercury).

Cardiac output and stroke volume were recorded by a well-validated noninvasive bioimpedance technique (NCCOM3; BoMed Medical Manufacturer Ltd).[30] These parameters were corrected for body surface area and described as cardiac index (CI, liters per minute per meters squared) and stroke index (SI, milliliters per meter squared).[2] Total peripheral vascular resistance index (TPVRI) was calculated as MAP divided by CI and expressed in arbitrary units (AU).

Study Design

Responses to BQ-788 (3, 30, and 300 nmol/min) and placebo were investigated in a placebo-controlled, four-way crossover study. Study drugs were administered in a single-blind manner. The order of treatments was randomized. Five subjects attended for 4 separate study visits, each separated by at least 5 days. Subjects were resident in the research center for at least 6 hours. Subjects rested supine for at least 20 minutes before hemodynamic measures, and baseline measures were made in the 30 minutes before study drug administration.

Analysis

Plasma ET-1 and big ET-1 are represented as absolute change from predose (picograms per milliliter), with statistical significance assessed by paired t test. Hemodynamic results are expressed as maximum placebo-corrected percentage changes from baseline ±SEM.[2] Statistical analysis was performed on untransformed data. Responses were examined by repeated-measures ANOVA. Statistical significance was taken at the 5% level, and analysis was performed using an Excel data analysis package (Excel 5.0, Microsoft Ltd).

Results

All 5 healthy male subjects (age range, 33 to 48 years) completed the study. No adverse events were reported, and there were no clinically relevant changes in routine biochemistry and hematology blood tests.

Plasma ET-1 and Big ET-1

Predose plasma ET-1 concentrations did not differ significantly for any of the treatments (range of baseline mean values, 4.4 to 4.9 pg/mL). Plasma ET-1 concentration increased significantly after administration of BQ-788 (from 4.6 ± 0.8 to 8.4 ± 1.8 pg/mL at 15 minutes with 300 nmol/min, $P = 0.02$) but not during treatment with the lower doses of BQ-788 or placebo (Figure 1). In contrast, concentrations of big ET-1 did not change significantly with treatment.

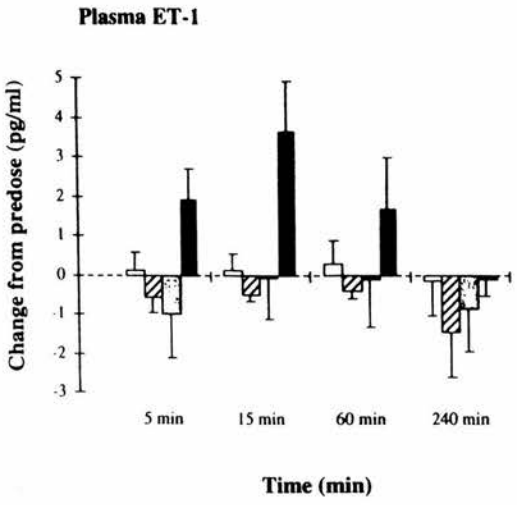

Figure 1. Change in plasma ET-1 concentrations after 15-minute intravenous infusion of BQ-788 or saline placebo in 5 subjects. Solid columns indicate BQ-788 at 300 nmol/min; shaded columns, BQ-788 at 30 nmol/min; hatched columns, BQ-788 at 3 nmol/min; and open columns, placebo. Plasma ET-1 concentrations increased significantly after administration of BQ-788 at 300 nmol/min.

Hemodynamic Parameters

Baseline measurements for hemodynamic parameters during the placebo treatment period were as follows: MAP, 79±3 mm Hg; HR, 79±3 bpm; CI, 2.6±0.2 (L/min)/m^2; SI, 49±3 mL/m^2; and TPVRI, 31.1±1.8 AU. Baseline values were similar for each of the other treatment periods. MAP did not alter significantly after administration of BQ-788 at any dose (3±2% at 90 minutes with 300 nmol/min; P=0.4) (Figure 2). After administration of BQ-788, there were changes in all other hemodynamic parameters when compared with placebo that appeared to be dose-related and that were significant at 300 nmol/min; HR decreased (13±7% at 50 minutes after dose; P=0.002), CI decreased (17±5% at 40 minutes; P<0.0001), and there was a small reduction in SI (8±4% at 40 minutes; P=0.002). TPVRI increased (24±5% at 40 minutes; P<0.0001).

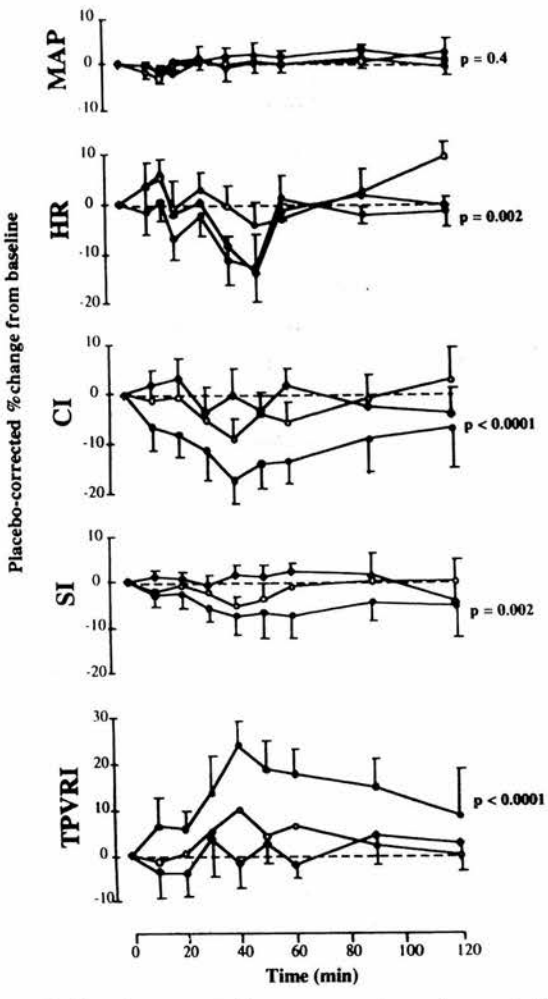

Figure 2. Placebo-corrected mean percentage change in MAP, HR, CI, SI, and TPVRI after 15-minute intravenous infusion of BQ-788 or saline placebo in 5 subjects. Closed circles indicate BQ-788 at 300 nmol/min; open circles, BQ-788 at 30 nmol/min; and shaded diamonds, BQ-788 at 3 nmol/min. There was no change in MAP, but there was a reduction in HR, CI, and SI and an increase in TPVRI after administration of BQ-788 at 300 nmol/min.

Discussion

We have demonstrated substantial systemic vasoconstriction, associated with a reduction in HR and CI but no change in MAP, in response to administration of the selective ET_B receptor antagonist BQ-788 in healthy men. Consistent with our earlier work in the forearm circulation,[3] these observations are highly suggestive of the overall effect of endogenous ET_B receptor–mediated vascular tone favoring vasodilatation. An alternative explanation for the hemodynamic effects is that BQ-788 is directly negatively chronotropic and that peripheral effects are indirect. However, this is unlikely given our earlier work[3] and the lack of evidence of an important positive chronotropic and inotropic role of the cardiac ET_B receptor.[14] Although peripheral resistance was substantially increased, blood pressure was unaffected because of a decrease in HR that was probably reflex in origin. We have also demonstrated increases in plasma ET-1, but not big ET-1, concentrations after ET_B receptor blockade, consistent with reduced clearance of ET-1 by the ET_B receptor.[15] All of these effects were prominent with BQ-788 at the highest dose but were not clearly seen at lower doses.

The vasoconstrictor effects of ET_B receptor antagonism may result directly from blockade of the vasodilator effects of the endothelial ET_B receptor or indirectly from displacement of endogenously generated ET-1 from ET_B receptors to unoccupied ET_A receptors. It is unlikely that these effects are mediated by nonselective ET_A/ET_B receptor blockade because they are the opposite of those found with selective ET_A receptor antagonists in healthy subjects (unpublished data, 1998) and patients with heart failure[22] and of those found with combined ET_A/ET_B receptor antagonists in healthy subjects.[2] Clearly, the indirect effects of ET-1 on ET_A receptors are more relevant with administration of selective ET_B antagonists than with nonselective ET_A/ET_B receptor antagonists, because in this latter situation the constrictor ET_A receptor is also blocked. Indeed, vasodilator effects have been demonstrated with both selective[1,3,23] and nonselective[2,24]

endothelin receptor antagonists in humans, and the nonselective ET_A/ET_B antagonist bosentan has recently been shown to effectively lower blood pressure in patients with hypertension.[25] However, direct comparison of the effects of selective and nonselective endothelin receptor antagonism will be important in assessing the relative contribution of each receptor subtype to the vascular effects of ET-1.

We and others have previously demonstrated forearm vasodilatation in response to local ET_A receptor antagonism with BQ-123.[1,3,32] In the presence of BQ-788 in healthy volunteers, this effect was attenuated,[3] suggesting that the overall effect of vascular ET_B receptor stimulation by endogenous ET-1 is vasodilatation. This attenuation of BQ-123–mediated vasodilatation by BQ-788 suggests that the vasoconstrictor effect of ET_B receptor blockade is not mediated by displacement of ET-1 onto the ET_A receptor but is due to direct blockade of ET_B–mediated vasodilator tone. We have also shown, using a "nitric oxide clamp" technique, that the vasodilator response to BQ-123 is in part mediated by nitric oxide[3] and, therefore, probably mediated by the endothelial ET_B receptor. Loss of endothelial cell ET_B–mediated vasodilator tone may occur in cardiovascular diseases, such as essential hypertension and hypercholesterolemia, in which there is associated endothelial dysfunction.[33,34] Here, because of a reduced capacity for ET_B receptor–mediated, nitric oxide–dependent dilatation, selective ET_A receptor antagonists may be less effective.

In summary, we have demonstrated systemic vasoconstriction in response to acute ET_B receptor blockade with the selective ET_B receptor antagonist BQ-788 in healthy men in vivo, indicating that the predominant endogenous effect of stimulating vascular ET_B receptors is vasodilatation. One exciting possibility is that tonic endogenous ET-1 release, acting via the endothelial ET_B receptor, is responsible for the physiological basal release of nitric oxide. This now needs to be addressed in clinical studies. Further investigation of the influence of ET_B receptor antagonism on the sympathetic nervous system and renal function are also warranted. In addition, direct comparison of the effects of chronic administration of selective ET_A and combined ET_A/ET_B receptor antagonists is required in patients with cardiovascular disease, with and without endothelial dysfunction, in order to confirm which of these approaches is likely to be more effective in the clinical setting.

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Chapter Six

The endothelin system: a novel therapeutic target in cardiovascular disease

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Summary

Endothelin-1 (ET-1) is a powerful vasoconstrictor peptide which is generated by the vascular endothelium. It was first described in 1988 by Yanagisawa and colleagues and has since become the focus of a great deal of research interest. As a result of its potent vasoconstrictor effects, ET-1 has been implicated as an important mediator of the raised peripheral vascular resistance associated with a number of cardiovascular conditions, notably heart failure, hypertension and renal failure. A number of endothelin receptor antagonists are currently in development as novel treatments for cardiovascular disease. These compounds have vasodilator effects in healthy volunteers and in patients with cardiovascular disease both in the presence and absence of existing treatments. Endothelin antagonists have also been shown to have beneficial effects on renal function and may be of value in the treatment of both chronic and acute renal failure.

Although current treatments have proven effective in limiting disease progression and mortality, cardiovascular disease remains a significant public healthcare problem. The endothelin system provides an exciting therapeutic target for the development of new treatments for a number of cardiovascular diseases. The results from ongoing clinical trials are awaited with interest.

Background

ET-1 is a potent vasoconstrictor peptide which is generated by the vascular endothelium [1]. ET-1 plays an important role in the regulation of vascular tone and blood pressure *in vivo* [2] and, as a result of its powerful and sustained vasoconstrictor effects [3], has been implicated as a mediator of the raised peripheral vascular resistance associated with a number of cardiovascular diseases, including essential (or primary) hypertension, chronic heart failure and chronic renal failure. In addition to its direct vasoconstrictor actions, ET-1 can potentiate the vasoconstrictor effects of other constrictor substances including noradrenaline and angiotensin II [4]. ET-1 has co-mitogenic effects [5], and thus may play a role in vascular modelling and cell proliferation. The direct and indirect effects of ET-1 on vascular function

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Figure 1: Endothelin-1 (ET-1) is a 21 amino acid generated by a precursor pathway through the actions of endothelin converting enzyme (ECE). A number of vasoactive factors are involved in the regulation of this important vasoconstrictor peptide [1].

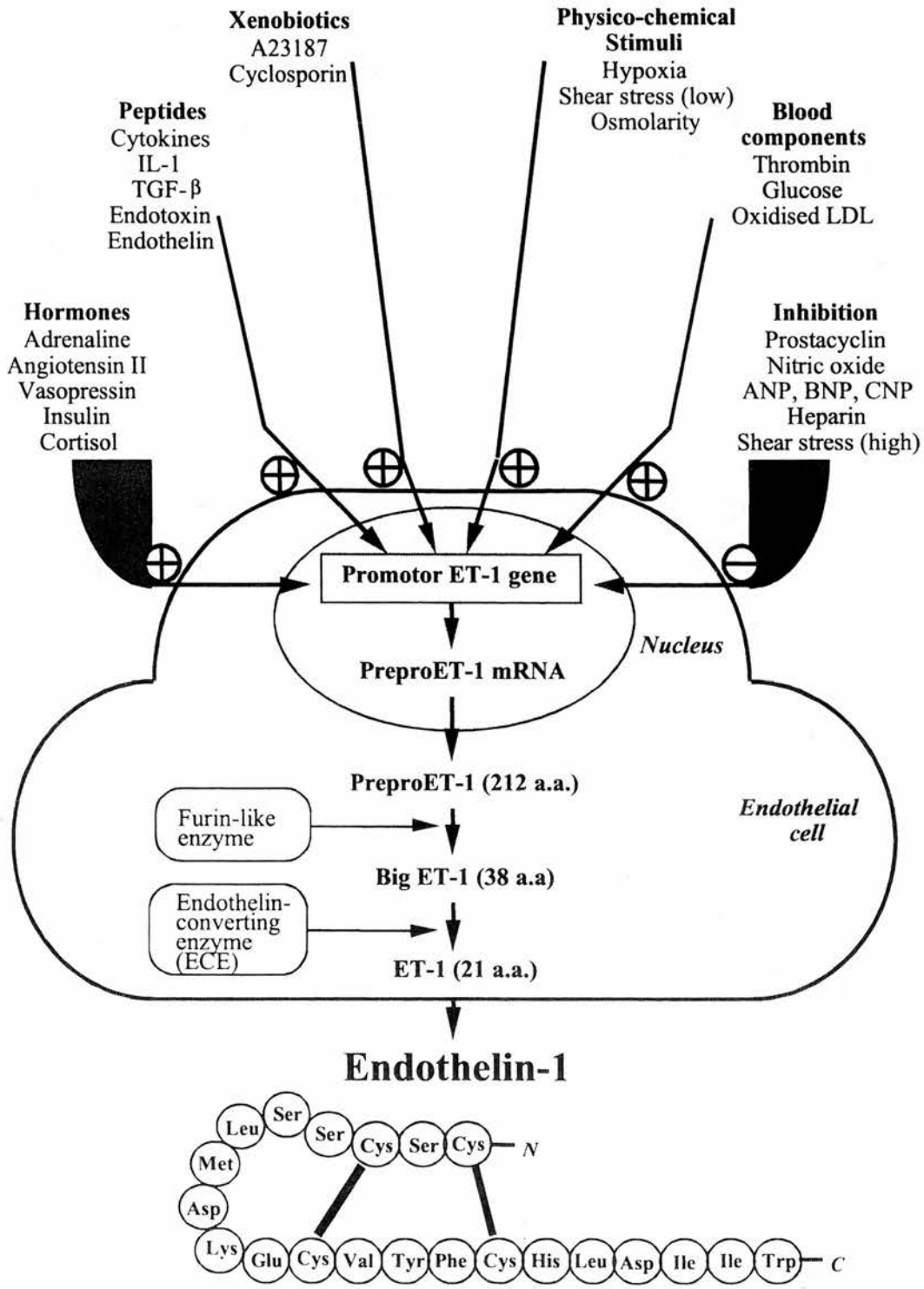
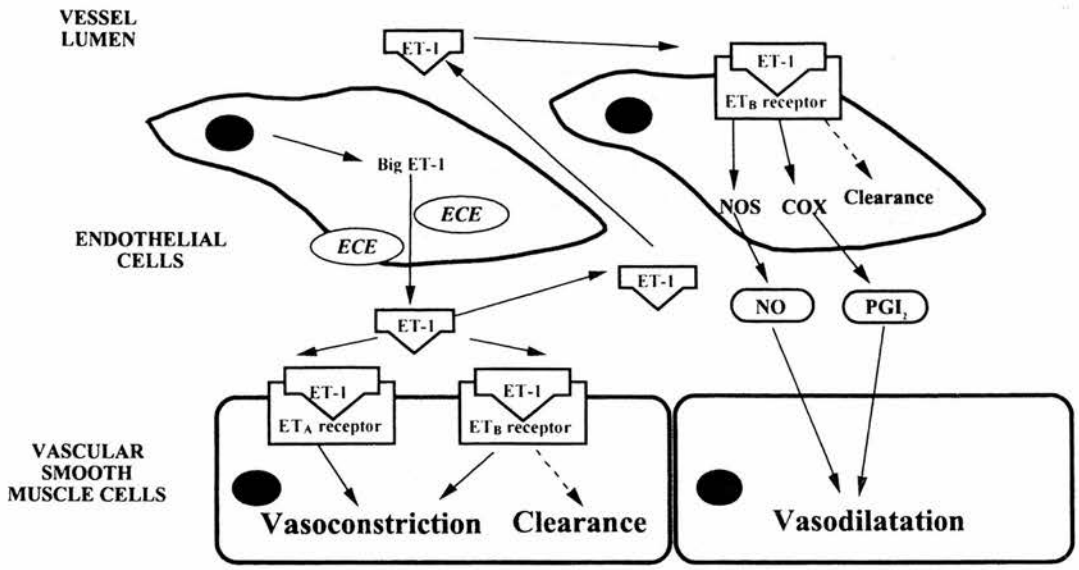


Figure 2: Endothelin-1 is generated by endothelial cells and is secreted abluminally to act on both ET_A and ET_B receptors. ET_A mediates the potent vasoconstrictor effects of endothelin-1. The ET_B receptor mediates vasodilatation through generation of nitric oxide and prostacyclin. The ET_B receptor may also mediate vasoconstriction and acts as a clearance receptor for endothelin-1. The balance of effects at the ET_B receptor appears to favour dilatation [85].
COX: Cyclo-oxygenase; ET-1: Endothelin-1; NO: Nitric oxide; NOS: Nitric oxide synthase; PGI_2: Prostacyclin.



indicate its potential importance in the pathophysiology of the development and progression of cardiovascular disease. Plasma concentrations of ET-1 are increased in a number of cardiovascular diseases, including heart failure [6,7], renal failure [8,9], and myocardial infarction (MI) [10], and, in most cases, the increase in plasma endothelin concentrations correlates with markers of disease progression. Although it is unclear whether the increase in plasma concentrations of ET-1 is merely a marker of disease progression, there is strong evidence to support a functional role for ET-1 in the pathophysiology of a number of cardiovascular diseases.

ET-1 is continuously generated from its precursor, big ET-1, through the actions of a specific endothelin converting enzyme (ECE) (**Figure 1**). A number of factors stimulate the generation of ET-1, including the vasoactive hormones, angiotensin II and arginine vasopressin [11], inflammatory mediators and physicochemical factors, such as altered vascular shear stress and hypoxia. ET-1 generation can also be inhibited by a number of vasoactive factors, including nitric oxide [12]. Two endothelin receptors mediate the vascular effects of ET-1 in humans, the ET_A [13] and ET_B [14] receptors (**Figure 2**). The ET_A receptor situated on the vascular smooth muscle mediates the potent vasoconstrictor effects of ET-1. The ET_B receptor is present on endothelial cells where it mediates vasodilatation through generation of nitric oxide and dilator prostanoids [15,16]. ET_B receptors have also been described on vascular smooth muscle cells [17] and these receptors appear, under some circumstances, to mediate vasoconstriction [18,19]. However, the contribution of the ET_B receptor to endogenous ET-1 mediated constrictor tone is currently unclear. From the results of recent studies it would appear that, in healthy blood vessels [20] and in the blood vessels of patients with chronic heart failure [21], the vasodilator effects of the ET_B receptor predominate. However, further clinical trials, in patients with other forms of cardiovascular disease, are required to confirm whether this is also true in a clinical setting. Clarification of

the overall balance of the vascular effects of ET_B receptor stimulation, and effects on other organs such as the kidney, will be crucial in determining whether combined $ET_{A/B}$ or selective receptor blockade will be more beneficial in the treatment of cardiovascular disease. A clearance role for the ET_B receptor has also been proposed [22,23]. Although clearance has been demonstrated from the lungs, liver and kidney [22], a specific site for the clearance ET_B receptor has not been identified. However, the clearance role of the ET_B receptor again provides a rationale for selective blockade of the ET_A receptor, to allow continuing clearance of endogenously generated ET-1 by the ET_B receptor. The development of ECE inhibitors and combined $ET_{A/B}$ and selective endothelin receptor antagonists has been helpful in characterising the endothelin system. As yet there are no selective ECE inhibitors in clinical development.

The potential value of endothelin antagonists as vasodilator treatments is currently being investigated. Although much of the research interest is focused on the potential of endothelin receptor antagonists in cardiovascular disease, endothelin receptor antagonists may also be of value in the treatment of a number of conditions as diverse as bronchospasm [24], prostate cancer [25], portal hypertension [26], and Gram-positive infection [27].

The focus of this review will be the development of endothelin antagonists as vasodilator and antiproliferative treatments in cardiovascular disease.

Medical need

Cardiovascular disease is one of the main causes of morbidity and mortality in Western society. Despite the development of effective therapies, primary cardiovascular diseases commonly progress to heart failure [28]. Recently successful therapies in chronic heart failure focus on inhibiting neurohumoral pathways and other vasoconstrictors, thereby reducing peripheral vascular resistance. Although this approach has proven successful in slowing disease progression and in improving both morbidity and mortality, chronic heart failure remains a significant cause of death and disability in the UK [29]. The endothelin system has emerged as an attractive new therapeutic target in cardiovascular medicine. We will discuss the potential of endothelin antagonists in the context of essential hypertension, chronic heart failure, acute and chronic renal failure, MI and subarachnoid haemorrhage.

Essential hypertension

Essential hypertension is a condition in which arterial pressure is increased with no apparent primary cause. Peripheral vascular resistance is increased, and vascular and cardiac hypertrophy, which are associated with a worse prognosis, may develop. Hypertension is commonly seen in the middle-aged and elderly, affecting one in seven of the adult population, and is associated with an increased risk of stroke, MI, heart failure and renal failure. The current aim of therapy is to reduce the degree of blood pressure within acceptable limits and thereby prevent the risk of cardiovascular complications. Changes in lifestyle and dietary habits can help reduce hypertension but drug intervention is required where these measures fail or where the degree of hypertension is more severe [30].

Plasma endothelin concentrations are not normally raised in essential hypertension but have been described in patients with co-existing renal disease [8]. Nevertheless, the potent vasoconstrictor and vasopressor actions of ET-1 may play a role in the development of increased blood pressure and in the pathophysiology of disease progression. The comitogenic properties of ET-1 may be important in the hypertrophic and atherosclerotic process, again associated with an increasing cardiovascular risk. Early studies with endothelin receptor antagonists in hypertension have been promising. Local vasodilatation has been demonstrated in response to the ET_A selective antagonist, BQ-123 [31], in healthy volunteers

[32] and in patients with hypertension [33]. Systemic administration of the combined $ET_{A/B}$ receptor antagonist, TAK-044 [34], lowered blood pressure and systemic vascular resistance in healthy volunteers [2]. Interestingly, a single oral dose of the combined $ET_{A/B}$ antagonist, bosentan, was effective in lowering blood pressure in patients with essential hypertension [35]. Unpublished data suggest a chronic effect on blood pressure of endothelin receptor antagonism with bosentan that is equivalent to that associated with the ACE inhibitor, enalapril. In essential hypertension, endothelin antagonists may provide additional blood pressure reduction in combination with existing therapies or may be useful in treating hypertension that has proven resistant to current methods of treatment. The combined vasoconstrictor and co-mitogenic properties of ET-1 provide an argument for the use of endothelin antagonists in this condition and the results of further studies are awaited.

Heart failure

Heart failure is a common and disabling condition that results from impaired left ventricular function. Cardiac output is lowered, sodium and water retention develop, and there is increased peripheral vascular resistance associated with activation of the sympathetic nervous system and the renin-angiotensin system [36,37]. A successful approach has been to reduce peripheral vascular resistance in order to lessen the damaging effects of increased vascular tone on cardiovascular function. Indeed, the use of ACE inhibitors [38], hydralazine or nitrates [39] has proven this approach to be effective in terms of both symptoms and prognosis. However, despite the apparent success of ACE inhibitors, mortality remains high [29]. The endothelin system may provide an additional therapeutic option in heart failure.

Plasma concentrations of ET-1 and its precursor, big ET-1, are elevated in heart failure and correlate with the likelihood of death and with the need for cardiac transplantation [6,7]. Although the increase in plasma ET-1 may result from reduced renal clearance, the increase in big ET-1 concentrations indicates a likely increase in the generation of ET-1. Given the potent vascular effects of ET-1, the correlation between measures of mortality and morbidity and the increase in plasma levels of ET-1 and big ET-1, the endothelin system is likely to be of functional importance in the pathophysiology of heart failure. Early clinical studies have demonstrated benefits of endothelin antagonists in the presence and absence of existing treatment with an ACE inhibitor. Local vasodilatation to selective ET_A antagonism with BQ-123 and to ECE inhibition with phosphoramidon, a non-selective ECE/NEP inhibitor [40], was shown in patients with heart failure who were receiving treatment with an ACE inhibitor [41]. Systemic administration of bosentan in patients with heart failure produced significant systemic and pulmonary vasodilatation; reducing mean arterial pressure, pulmonary arterial pressure, right atrial pressure and pulmonary artery wedge pressure. Cardiac index was also increased with no change in heart rate [42]. In healthy human vasculature it would appear that there is an endogenous ET_B-mediated dilator tone [20]. Interestingly, in patients with heart failure, selective ET_B receptor antagonism with BQ-788 [43] resulted in local vasoconstriction [21], indicating that, despite increased plasma concentrations of ET-1, the endothelial ET_B receptor provides sufficient vasodilator effects to balance the constrictor effects of the vascular smooth muscle ET_B receptor. Further investigation of the systemic effects of combined $ET_{A/B}$ and selective endothelin receptor antagonism in heart failure is required to confirm which approach will be of more benefit in the clinical setting. Phase II and III trials investigating selective and combined $ET_{A/B}$ endothelin antagonists in the treatment of heart failure are in progress.

Given the increase in plasma concentrations of big ET-1 in heart failure [6,7] and the proposal that this indicates an increase in the generation of ET-1, ECE inhibitors may also be of value in treating chronic heart failure. As yet there are no selective ECE inhibitors in clinical development but non-selective ECE/NEP inhibitors are being investigated in this context.

Chronic renal failure

Chronic renal failure results from a progressive loss of renal function, the two commonest causes being hypertension and diabetes mellitus [44]. Continued loss of renal function despite the absence of underlying disease activity indicates that progressive damage results from common mechanisms independent of the initial cause. Chronic renal vasoconstriction contributes to the loss of functioning renal tissue and, although no specific mediator has emerged as a clear therapeutic target, blood pressure control has proven to be of value in the treatment of renal failure [45]. There is no clear evidence that one class of antihypertensive agent is better than another [46]. However, ACE inhibitors have demonstrated renoprotective effects in diabetes mellitus [47] and have proven useful in lowering blood pressure in this context. There are a number of potential mediators involved in renal vasoconstriction and long-term studies comparing the effects of ACE inhibitors with alternative therapeutic measures are necessary.

Plasma concentrations of ET-1 are elevated in renal disease [8,9] and are likely to result from a reduced renal clearance of ET-1 rather than increased production of the peptide. However, it is also likely that ET-1 is involved in the progression of renal disease [48]. Indeed, in a rat model of progressive renal disease, renal ET-1 gene expression correlated with disease progression [49]. From animal studies, there is increasing evidence that ET-1 plays a role in the progression of renal disease. Investigation of the role of ET-1 and the endothelin receptor subtypes in renal function and the progression of renal disease in humans is ongoing.

Although the ET_B receptor subtype predominates in the human kidney [50], a greater proportion of ET_A receptors are localised to the vasculature and the ET_A subtype is more likely to be important in mediating renal vasoconstriction to ET-1 [51]. The selective ET_A receptor antagonist, FR139317, appeared to be successful in preventing disease progression in a rat model of chronic renal disease; with a reduction in proteinuria, markers of cell proliferation and evidence of protection against glomerular structural injury [52]. Tubular ET_B receptors may offer renoprotection through sodium and water excretion in ischaemic conditions [53]. Therefore, it may be more important to selectively block the ET_A receptor and preserve ET_B mediated diuresis and natriuresis [53]. It is important to interpret the results of studies in animal models with caution, due to species differences in renal physiology [45] and in endothelin receptor distribution [53]. Early clinical trials have been conducted in patients with chronic renal failure. The results of such studies will help identify the potential of endothelin antagonists in a clinical context.

Acute renal failure

Acute renal failure develops over a period of hours to days and can result from tubular ischaemia, obstruction or toxic insult, or from a marked reduction in renal perfusion [54]. Acute renal failure is associated with a high mortality [55] and is a common complication of cardiac surgery and administration of radiocontrast agents. Renal vasoconstriction is thought to be important in the pathophysiology of radiocontrast nephropathy, and this may be mediated by ET-1 [56]. Indeed, plasma endothelin concentrations have been shown to increase following intravascular administration of a radiocontrast agent in rats [57], and selective ET_A antagonism reduced necrotic damage following radiocontrast administration in a rat model [58]. The potent vasoconstrictor effects of ET-1, combined with its effects on renal blood flow and glomerular filtration, suggest a role for endothelin in the pathophysiology of ischaemic acute renal failure [59,60]. In addition to the beneficial effects described in chronic renal failure, selective and combined $ET_{A/B}$ endothelin antagonists have also been shown to limit disease progression in acute models of renal failure [48,59,61,62]. Interestingly, administration of the ECE/NEP inhibitor, phosphoramidon [40], was more effective in restoring renal function and in minimising structural changes than selective ET_A antagonism with BMS-182874 in a model of ischaemic renal failure [63]. Again, further studies and direct

comparison of selective and combined $ET_{A/B}$ antagonists are required to confirm the potential of these compounds in the clinical setting.

Myocardial infarction

Plasma ET-1 concentrations are increased in MI and are strongly related to mortality [10]. The vascular effects of ET-1 indicate a potential role in the progression of myocardial damage to heart failure. However, it is not clear whether ET-1 plays a role in infarct development. A number of studies have been carried out in animal models with conflicting results. In a model of MI in pigs, the selective ET_A receptor antagonist, PD 1567807, did not reduce infarct size [64]. In contrast, the selective ET_A receptor antagonist, BQ-123, improved survival and lessened cardiac dysfunction in a rat model of MI [65]. These differences may be explained by species difference in receptor distribution and function but may also be related to the timing of drug administration in relation to the myocardial event. The potential of endothelin antagonists in the treatment of MI and the prevention of the progression of cardiovascular disease remains to be confirmed.

Subarachnoid haemorrhage

Subarachnoid haemorrhage carries a risk of rebleeding and of cerebral ischaemia associated with cerebral vasospasm. Although the mechanism involved in cerebral vasospasm is unclear, ET-1 is a likely mediator of this effect [66]. In support of this, combined $ET_{A/B}$ antagonism with bosentan was effective in preventing [67] and reversing [66] cerebral vasospasm in a rabbit model of subarachnoid haemorrhage. Selective ET_A receptor antagonism, with BQ-485 [68] and with Ro 61-1790 [69], is also effective in limiting cerebral vasospasm in a dog model of subarachnoid haemorrhage. Clinical trials investigating the effects of bosentan in patients with subarachnoid haemorrhage are in progress and the results have so far been promising [Breu, 1997 - personal communication]. ET-1 may also be involved in the development of cerebral ischaemia through cerebral vasoconstriction. Further investigation of selective and combined $ET_{A/B}$ endothelin antagonists in the treatment of subarachnoid haemorrhage will reveal the true potential of these compounds in this context.

Existing treatment

Essential hypertension

Diuretics and beta-blockers are currently recommended as the treatments of first choice in essential hypertension [30,70]. Clinical trials with diuretics and beta-blockers show they reduce the risk of cardiovascular complications, including stroke, coronary heart disease and renal disease [70], but these drugs have well documented limitations [30].

Although diuretics and beta-blockers are traditionally the first line of drug treatment in hypertension, additional classes of drugs are recommended for cases resistant to this approach. These additional drugs include ACE inhibitors, angiotensin II receptor antagonists, calcium antagonists and alpha-blockers [30,70]. It is common for drug treatments to be given in combination, especially in cases that have only partially responded to initial treatment.

ACE inhibitors are indicated in patients with heart failure or with diabetic nephropathy [71] and have been shown to reduce albuminuria in patients with hypertension [72]. Although ACE inhibitors have demonstrated renoprotective effects [47] through a lowering of intraglomerular pressure, this can unacceptably lower glomerular filtration rate in patients with renal artery stenosis. Therefore, ACE inhibitors should be prescribed with caution in this group. ACE inhibition leads to an increase in tissue kinin levels which may lead to symptoms of cough, urticaria and angioneurotic oedema, and these adverse effects have obvious effects on patient compliance and tolerance of this treatment.

Angiotensin II receptor antagonists are a relatively new class of compounds that block the effects of angiotensin II without blocking its generation. There is no associated accumulation of kinins and, consequently, the kinin mediated adverse effects associated with ACE inhibition are absent. However, beneficial kinin-mediated dilatation may also be lost [70].

Calcium antagonists are also prescribed in the treatment of hypertension. Although administration of these compounds has been associated with some disadvantages, results from the SYST-EUR trial in elderly patients suggest that there is a reduction in the incidence of stroke and in cardiovascular-related mortality with long-term administration of the calcium antagonist nitrendipine [73].

Despite increasing knowledge of the risk factors associated with the development of hypertension and the mechanisms underlying increased vascular tone, a considerable proportion of hypertension remains resistant to current treatments [30,70]. One explanation for this could be poor patient compliance. Also, it may be true that there are a number of different mediators of hypertension and that the current range of therapies, although broad, is not exhaustive and is not directed at causal influences. A number of genotypic variants have been described which could identify individuals with differences in metabolism or responses to a given class of drug [74]. If proven reliable, treatments could in future be prescribed according to specific genotypes.

Heart failure

Treatment of heart failure should provide symptomatic relief, aim to prevent or delay the progression of left ventricular dysfunction, and reduce mortality [39]. This requires a combination of drugs, currently including: diuretics, vasodilators - nitrates, ACE inhibitors, hydralazine and isosorbide dinitrate - digoxin, anti-arrhythmic drugs and anticoagulants. Of these drugs, ACE inhibitors have been the most effective in reducing both morbidity and mortality in patients with heart failure [38]. The therapeutic benefits of ACE inhibition probably result from a combination of vascular effects: prevention of the constrictor effects of angiotensin II, increased concentrations of the vasodilators bradykinin and nitric oxide, and sympatholytic and parasympathomimetic effects. However, despite the success of ACE inhibition, mortality remains high. Heart failure still represents a large public health problem in the US, with approximately 400,000 new cases reported annually and annual healthcare costs currently quoted at $17.8 billion [28]. Similarly, heart failure is a growing problem in the UK with a reported annual cost to the NHS of £360 million [75].

Chronic renal failure

The aim of treatment in chronic renal failure is to slow its progression to prevent end-stage renal disease. Current therapeutic approaches involve maintenance of blood pressure within acceptable limits, renoprotection with ACE inhibitors, dietary restriction, close monitoring of renal function and avoidance of further renal insult [44]. Loop diuretics are effective in reducing oedema associated with chronic renal failure [76]. There is a limited range of effective therapies to prevent progression of chronic renal disease, and the health cost is high. Although ACE inhibitors have proved useful in this context in terms of lowering blood pressure and in reducing proteinuria, clinical studies comparing other treatments are required to confirm that that their popularity matches their efficacy [46]. The limitations of ACE inhibitor therapy described in the sections above illustrate the need for additional therapies for use in combination with, or in place of, ACE inhibitors.

Acute renal failure

Initial treatment of acute renal failure focuses on treatment of the underlying condition and correction of fluid and electrolyte imbalances. Haemodialysis is indicated in severe cases of acute renal failure [54]. As yet, there is no clear drug of choice for acute renal failure and

treatments are aimed at support and prevention of further renal damage. Loop diuretics may be prescribed in acute renal failure. However, their benefits have not been confirmed and long-term studies are in progress to evaluate their effects [76]. Dopamine has also been indicated in the prevention and treatment of acute renal failure. However, as there is no clear cut evidence to support its efficacy in this context, long-term trials are required [77]. The cost of dialysis is considerable and the development of less expensive and more effective forms of treatment for acute renal failure is of obvious advantage.

A number of vascular mediators are being investigated as potential therapeutic targets in acute renal failure. Atrial natriuretic peptide (ANP) is an endogenous hormone that increases glomerular filtration rate, blocks tubular reabsorption of sodium and chloride, and reverses endothelin-induced vasoconstriction. In a study of patients with renal dysfunction following cardiac surgery, treatment with ANP improved renal function and reduced renal vascular resistance [78]. However, acute treatment with anaritide, a synthetic form of ANP, did not improve overall mortality rates in patients with acute tubular necrosis [79]. The anti-inflammatory and antiproliferative effects of peptides isolated from matrix proteins with the Arg-Gly-Asp sequence (RGD peptides) have generated interest in these compounds as potential new treatments for acute renal failure [80].

Myocardial infarction

Early reperfusion therapy in acute MI, through thrombolysis with pharmacological agents or direct angioplasty, improves survival rates. However, reperfusion injury can occur and complicate the recovery process. Clinical trials investigating the need to protect the myocardium against reperfusion injury with agents such as adenosine are ongoing [81]. ACE inhibitors are also indicated following MI, especially in patients with left ventricular dysfunction. In the SAVE trial [82], long-term treatment with the ACE inhibitor, captopril, within 3 - 16 days following MI improved survival and reduced the development of heart failure in patients with asymptomatic left ventricular dysfunction. However, despite improvement in mortality and delay in the progression of heart failure with current treatments, myocardial infarction is still associated with significant morbidity and mortality. The timing of drug treatment in myocardial infarction can have a marked effect on the success of the intervention as early initiation of ACE inhibition may lessen the associated therapeutic benefits [83]. Further investigation of the pathophysiology of the remodelling process is required.

Subarachnoid haemorrhage

The development of cerebral vasospasm following subarachnoid haemorrhage is associated with much of its mortality and morbidity. However, there is no clear drug of choice recommended for the prevention of this event. Rebleeding and subsequent cerebral infarction is a risk associated with subarachnoid haemorrhage. Treatment with the calcium antagonist, nimodipine, has been shown to reduce the incidence of cerebral infarction and improve outcome following subarachnoid haemorrhage [84], although to a limited degree.

Current research goals

The therapeutic potential of a number of orally active endothelin receptor antagonists is currently under investigation in Phase II and Phase III clinical trials in heart failure and in hypertension. Demonstration of a vasodilator response to these compounds, especially in the presence of existing therapies, will be vital if endothelin antagonists are to be indicated as alternative treatments in these chronic cardiovascular conditions. Once initial benefits have been demonstrated, long-term trials will be necessary to confirm their benefits in terms of prevention of disease progression. Phase II and III clinical trials investigating the long-term administration of orally-active compounds in the treatment of patients with heart failure are

in progress. Intravenous compounds are currently being investigated as potential treatments in acute renal failure. Studies in animal models have proven promising and clinical trials are now required to confirm the potential of these compounds. The question of whether combined $ET_{A/B}$ or selective receptor antagonism will be of more benefit as vasodilator treatments depends on the balance between the ET_B receptor-mediated effects of vasodilatation, vasoconstriction and clearance [85]. Further investigation of the actions mediated by the ET_B receptor in health and in specific disease states is necessary to answer this question. This issue is of particular relevance in cardiovascular diseases, such as essential hypertension and in hypercholesterolaemia, where there is associated endothelial dysfunction [86,87], and there may be a reduced capacity for ET_B receptor-mediated dilatation.

ECE inhibition may be a useful approach in limiting the vascular effects of ET-1 in conditions where its generation is increased. ECE inhibitors are likely to have systemic vasodilator effects: directly by inhibition of ET-1 generation and consequent removal of its constrictor tone, and indirectly by removal of the inhibitory effects of ET-1 on nitric oxide generation [12]. Indeed, local vasodilatation was demonstrated following local administration of the non-selective ECE/NEP inhibitor, phosphoramidon [40], in humans *in vivo* [32]. At present there are no selective ECE inhibitors in clinical development, although ECE inhibitors are currently in preclinical development and are likely to progress towards studies in man.

Scientific rationale

ET-1 is a potent vasoconstrictor peptide generated by the endothelium [1]. It is one of a family of three related peptides: ET-1, ET-2 and ET-3, encoded from three distinct genes [88]. As ET-1 is the predominant isoform produced by the endothelium, it is likely to be the most important in the regulation of vascular tone. ET-1 is generated from its precursor, big ET-1, through the actions of an ECE. Generation of ET-1 is regulated at a transcriptional level and is influenced by a number of factors (**Figure 1**). At least two ECE isoforms have been identified [89,90], with a number of subtypes already described. It is not yet clear which of these isoforms is involved in the pathophysiology of cardiovascular diseases [85,91].

ET-1 is secreted abluminally to act on the vascular smooth muscle in a paracrine and autocrine manner. As a result, plasma concentrations of endothelin are not always an accurate reflection of functional activity. Two human endothelin receptors have been characterised: the ET-1-selective ET_A receptor [13] and the non-isoform-selective ET_B receptor [14] (**Figure 2**). The characteristically sustained vasoconstriction to ET-1 [3] is primarily mediated by the vascular smooth muscle ET_A receptor. The endothelial ET_B receptor stimulates generation of nitric oxide and dilator prostaglandins [15,16] to mediate vasodilatation to modulate the constrictor effects of the ET_A receptor. The ET_B receptor has also been described on the vascular smooth muscle [17], and, although the vascular smooth muscle ET_B receptor appears to mediate vasoconstriction under some circumstances [18,19], the physiological relevance of this effect remains to be proven. Indeed, it would appear that selective ET_B receptor antagonism results in systemic [92] and local [20,21] vasoconstriction, indicating that the balance of effects at the ET_B receptor favours vasodilatation [85]. The ET_B receptor is also thought to be involved in clearance of endothelin from the circulation [22]. In support of this, plasma concentrations of ET-1 are dose-dependently increased following administration of combined $ET_{A/B}$ [2,93,94] and selective ET_B [22,95] receptor antagonists but not following administration of a selective ET_A receptor antagonist [95]. It could be that the vasoconstrictor effects of selective ET_B receptor antagonism result partly from inhibition of ET_B-mediated clearance of ET-1, allowing the uncleared peptide to bind to unoccupied constrictor ET_A receptors [92].

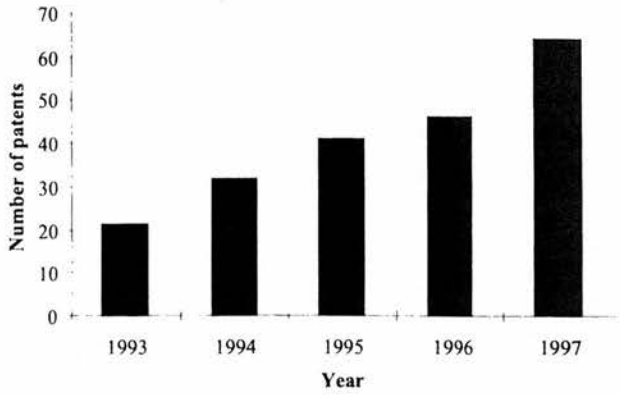


Figure 3: Trends in endothelin antagonist patenting 1993-1997. Data derived from MDL's ISISBase.

The balance between the constrictor effects of ET-1, mediated by the vascular smooth muscle ET_A and ET_B receptors, and the dilator effects, mediated by the endothelial ET_B receptor, is important in determining the value of combined $ET_{A/B}$ and selective endothelin receptor antagonists. As discussed earlier, there may be alterations in endothelin receptor function, distribution and sensitivity in disease states that could lead to an imbalance between the actions of ET-1 on the ET_A and ET_B receptors. Further investigation of the endogenous effects of endothelin receptor stimulation in health, and in disease development and progression, is required to identify the best approach to inhibit the damaging effects of chronic vasoconstriction or vasospasm while preserving the beneficial dilator effects and clearance actions of the endothelial ET_B receptor.

Competitive environment

There are currently a number of orally-active and intravenous endothelin antagonists in clinical development. The first of these compounds was bosentan, an orally active combined $ET_{A/B}$ receptor antagonist, which is already in long-term Phase III clinical trials investigating morbidity and mortality in patients with chronic heart failure. Although bosentan is currently placed to be the first endothelin antagonist to reach the market, a number of competitor compounds are also in Phase II and III trials. The orally active compounds will be valuable in the long-term treatment of chronic heart failure, hypertension and chronic renal failure. Of these, chronic heart failure is currently the favoured indication. Intravenous antagonists are likely to be indicated in the initial treatment of acute renal failure, MI and, if early clinical trials are encouraging, subarachnoid haemorrhage. Pulmonary hypertension, migraine and prostatic hypertrophy are additional indications for which these compounds may be considered.

Figure 3 illustrates the trends in pharmaceutical patenting of endothelin antagonists over the period 1993 - 1997. **Table 1** provides details of selected lead endothelin antagonists that have reached Phase II in their clinical development.

A number of pharmaceutical companies, including Abbott, Bristol-Myers Squibb, Knoll, Merck, Parke-Davis, and Zeneca, are developing a range of selective and combined endothelin receptor antagonists. From the currently available literature, there is no indication which of these compounds will be the company's lead compound or of the stage that they have reached in their clinical development.

Table 1: Competitive environment: selected lead endothelin antagonists in development.

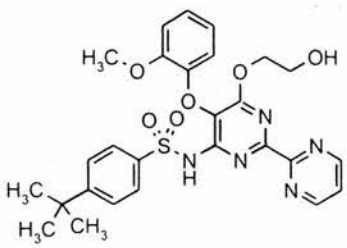
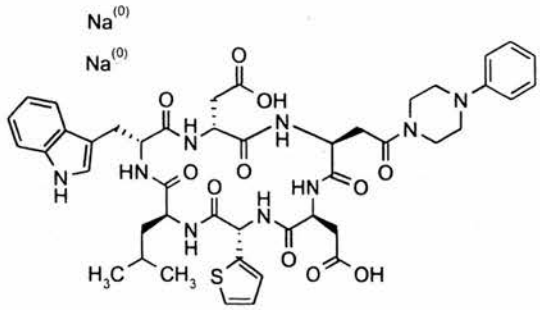
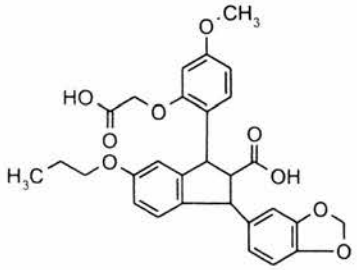
Company	Hoffmann-La Roche	Takeda
Product	Bosentan (Ro 47-0203)	TAK-044
Structure		
Phase	Phase III (cardiac failure)	Phase II - Japan (hypertension and myocardial infarction); Phase I - Europe (renal failure)
Patent **Priority**	EP-526708 12/05/92	
Source	ID*db*	ID*db*
Comments	Orally active, combined $ET_{A/B}$ receptor antagonist.	Intravenous compound, combined $ET_{A/B}$ receptor antagonist.

Table 1: Competitive environment: selected lead endothelin antagonists in development (*continued*).

Company	SmithKline Beecham	Texas Biotech
Product	SB-209670	TBC-11251
Structure		
Phase	Phase II (renal failure)	Phase IIa (congestive heart failure)
Patent **Priority**	WO9308799 20/03/92	
Source	ID*db*	ID*db*
Comments	Non-peptide compound, combined $ET_{A/B}$ receptor antagonist.	Non-peptide, orally active selective ET_A receptor antagonist.

In addition to endothelin receptor antagonists, compounds that inhibit the activity of the ECE are currently in preclinical development. As yet no selective compound is available for clinical use but some have been described that are likely to proceed to clinical trials within the next two years [96]. Also of interest are compounds with combined enzyme inhibitory actions. A dual inhibitor of ECE and ACE, SCH-54470, is currently in preclinical development as a potential treatment for renal failure [97]. Dual ET_A and angiotensin II receptor antagonists have also been described [98]. Given the benefits of separate inhibition of the endothelin and angiotensin systems, and the evidence that these systems are at least additive in heart failure [99], combined inhibition of these systems offers an attractive therapeutic option.

Potential development issues

The pharmaceutical market for the treatment of cardiovascular diseases has recently been dominated by ACE inhibitors, particularly in chronic heart failure and chronic renal failure. However, despite their success, there remain considerable mortality and morbidity associated with the cardiovascular diseases discussed in this review. Endothelin antagonists appear to offer additional vasodilatation in the presence of existing treatment with ACE inhibitors in patients with chronic heart failure [41]. This, combined with the effects of endothelin antagonism on renal function, highlights the potential benefits of inhibition of the actions of ET-1 in the treatment of the symptoms of cardiovascular disease and in slowing disease progression.

Common adverse events experienced by subjects in clinical trials with endothelin antagonists are consistent with systemic vasodilatation and include headaches, light-headedness and nausea. The fact that much of the vasodilatation to selective ET_A receptor antagonism is caused by nitric oxide [20] could indicate that tolerance to these effects will develop. Interestingly, these headaches have been a less frequent occurrence in clinical trials in patient groups than in healthy volunteer studies.

ECE inhibitors may prove useful in conditions where an increase in ET-1 generation is demonstrated and is of pathophysiological importance. These compounds would reduce the damaging effects of ET-1 and have the same advantages and disadvantages of combined $ET_{A/B}$ receptor antagonism. However, there are, as yet, no selective ECE inhibitors in clinical development and so these compounds are unlikely to be available for patient administration within the next two years.

Editorial analysis

ET-1 is an endothelium-derived vasoconstrictor and co-mitogenic agent which acts as a local paracrine and autocrine mediator, and is the most potent and sustained vasoconstrictor and pressor substance yet identified. ET-1 is now known to play an important physiological role in maintaining peripheral vascular tone and blood pressure [2,32]. ET-1 also has actions that might influence the function of the heart, kidney and nervous system. However, its physiological importance in these systems remains to be determined.

Abnormalities of the endothelin system are now recognised to occur in a range of diseases associated with vasoconstriction, vasospasm and vascular hypertrophy, and it appears that ET-1 may be causal, or at least contributory, in some of these pathophysiological processes. The use of endothelin receptor antagonists in experimental models of cardiovascular disease and in human clinical pharmacology studies has indicated a number of conditions - including hypertension, heart failure, acute renal failure, subarachnoid haemorrhage and pulmonary hypertension - in which further clinical studies would be worthwhile. A number of peptide and orally-active non-peptide endothelin receptor antagonists are now under clinical investigation and further studies are now required in specific diseases to determine whether selective ET_A or combined $ET_{A/B}$ receptor antagonists will be more effective.

Early clinical trials have demonstrated the potential therapeutic benefits of endothelin receptor antagonists in a number of cardiovascular diseases. These compounds appear to be well-tolerated, and effective, orally-active and intravenous formulations have been developed. The fact that additional benefits have been seen in the presence of existing treatments is encouraging for the future development of these compounds. Given the evidence in support of an important role for ET-1 in the pathophysiology of cardiovascular disease and the promising results of early clinical trials with endothelin receptor antagonists, treatments

that inhibit the effects of the endothelin system are likely to be of great importance in this clinical context.

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Endothelin-A Receptor Antagonist–Mediated Vasodilatation Is Attenuated by Inhibition of Nitric Oxide Synthesis and by Endothelin-B Receptor Blockade

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Background—The role of endothelin (ET)-1 in maintenance of basal vascular tone has been demonstrated by local and systemic vasodilatation to endothelin receptor antagonists in humans. Although the constrictor effects mediated by the vascular smooth muscle ET_A receptors are clear, the contribution from endothelial and vascular smooth muscle ET_B receptors remains to be defined. The present study, in human forearm resistance vessels in vivo, was designed to further investigate the physiological function of ET_A and ET_B receptor subtypes in human blood vessels and determine the mechanism underlying the vasodilatation to the ET_A-selective receptor antagonist BQ-123.

Methods and Results—Two studies were performed, each in groups of eight healthy subjects. Brachial artery infusion of BQ-123 caused significant forearm vasodilatation in both studies. This vasodilatation was reduced by 95% ($P=.006$) with inhibition of the endogenous generation of nitric oxide and by 38% ($P<.001$) with coinfusion of the ET_B receptor antagonist BQ-788. In contrast, inhibition of prostanoid generation did not affect the response to BQ-123. Infusion of BQ-788 alone produced a 20% reduction in forearm blood flow ($P<.001$).

Conclusions—Selective ET_A receptor antagonism causes vasodilatation of human forearm resistance vessels in vivo. This response appears to result in major part from an increase in nitric oxide generation. ET_B receptor antagonism either alone or on a background of ET_A antagonism causes local vasoconstriction, indicating that ET_B receptors in blood vessels respond to ET-1 predominantly by causing vasodilatation. **(*Circulation*. 1998;97:752-756.)**

Key Words: endothelin ■ nitric oxide ■ flow ■ receptors ■ prostaglandins

The endothelin (ET) family of peptides (ET-1, ET-2, ET-3) are generated in a variety of tissues and act primarily as paracrine and autocrine factors. The major isoform in the cardiovascular system, ET-1, is generated in the endothelium from a precursor, big ET-1, through cleavage by a specific endothelin-converting enzyme (ECE). Its actions are mediated by two receptors, the ET_A and the ET_B receptor, which have been characterized and cloned[1,2] and are pharmacologically distinct. The ET_A receptor has a higher affinity for ET-1 (ET-1≫ET-3), whereas the ET_B receptor is nonisopeptide selective (ET-1=ET-3). ET_A receptors are expressed on vascular smooth muscle cells, and their activation by ET-1 leads to vasoconstriction. The physiological importance of endogenous ET-1 in the maintenance of basal vascular tone and blood pressure in humans has been demonstrated by local[3,4] and systemic[4] vasodilatation in response to inhibitors of the endothelin system. An important role for the ET_A receptor in mediating this response is suggested by the substantial forearm vasodilatation to local administration of the selective ET_A receptor antagonist BQ-123[5] in healthy subjects.

Initially, it was thought that ET_B receptors were present only on endothelial cells, where they cause vasodilatation through release of endothelium-derived vasodilators, including nitric oxide (NO) and prostacyclin.[6,7] However, it is now recognized that ET_B receptors are also present on the smooth muscle of human arteries[8] and can mediate vasoconstriction,[9–11] although their contribution to ET-1–mediated constriction in humans remains to be defined.[12] Therefore, although ET_A receptor–mediated vasoconstriction is undisputed, it is unclear whether the balance of the effects of endogenous ET-1 on the endothelial and vascular smooth muscle ET_B receptors results predominantly in a vasodilator or constrictor tone.

In addition to mediating vasodilator effects of endothelial ET_B receptor activation, endothelium-derived dilators can in turn modulate the production and actions of ET-1.[9,13–15] In the short term NO inhibits production of ET-1[13] whereas chronic

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exposure causes upregulation of ET_A receptors.[16] In addition, endothelin receptor antagonists attenuate the pressor response to NO inhibition,[17,18] suggesting that this response may not simply be due to loss of basal NO-mediated dilator tone. These interactions indicate the existence of a complex relationship between the endothelin and NO systems.

As a consequence of its potent vasoconstrictor[19] and growth-promoting properties,[20] ET-1 has also been implicated in the pathophysiology of diseases such as hypertension, heart failure, and renal failure.[21] The recognition of the endothelin system as a new therapeutic target in the treatment of cardiovascular disease has lead to the rapid development of pharmacological agents that inhibit either the production of ET-1 or its actions. Recently, potent intravenous and orally active endothelin receptor antagonists with different pharmacological profiles have become available for clinical studies.[21,22] We are now in a position where it would be valuable to explore the contribution of the ET_B receptor to the vascular effects of ET-1.

The present study, in human forearm resistance vessels in vivo, was designed to further investigate the physiological role of ET_A and ET_B receptor subtypes and their possible interactions in mediating the vasodilator response to selective ET_A receptor antagonism. The first part of the study aimed to investigate whether increased release of the endothelium-dependent relaxant factors NO and prostacyclin contributes to the vasodilator response to selective ET_A receptor antagonism. We therefore compared the response to the selective ET_A receptor antagonist BQ-123 during local inhibition of NO synthase and during systemic inhibition of prostanoid generation with the response to BQ-123 alone. In the second part of the study, to investigate the role of the ET_B receptor in BQ-123–induced vasodilatation, we examined the effects of simultaneous ET_A and ET_B receptor blockade compared with ET_A or ET_B receptor blockade alone.

Methods

Subjects

Twenty-two healthy subjects (1 woman) ranging in age from 20 to 43 years participated in two studies that were performed in the University Hospital Utrecht (study 1) and the University Department of Medicine, Western General Hospital, Edinburgh (study 2), with the approval of the local research ethics committees of each hospital and the written informed consent of each subject. The investigations conformed with the principles outlined in the Declaration of Helsinki. No subjects had received vasoactive medication or nonsteroidal anti-inflammatory drugs within the week before each phase of a study, and all subjects abstained from alcohol for 24 hours and from food, caffeine-containing drinks, and tobacco for at least 4 hours before any measurements were made. All studies were performed in a quiet room maintained at a controlled temperature between 22°C and 24.5°C.

Drug Administration

The brachial artery of the nondominant arm was cannulated with a 22 (study 1) or 27 SWG cannula (study 2) under lidocaine local anesthesia (lidocaine 2%; Astra Pharmaceuticals Ltd). Drugs, with the exception of aspirin, were dissolved in physiological saline (0.9%, Baxter Healthcare Ltd) and infused intra-arterially at locally active doses. The infusion rate was kept constant at 80 mL/h (study 1) or 60 mL/h (study 2). All solutions were prepared aseptically from sterile stock solutions or ampules on the day of the study.

Drugs

BQ-123 (100 nmol/min, study 1; 10 nmol/min, study 2), was used as a selective ET_A receptor antagonist (study 1: American Peptide Co; study 2: Clinalfa AG). We have demonstrated previously local forearm vasodilatation to intra-arterial infusion of BQ-123 (100 nmol/min).[3] In study 2, we used a 10-fold lower dose of BQ-123 (10 nmol/min) because more recent studies have shown that this causes vasodilatation of equal magnitude to that seen with the higher dose.[23] BQ-788 (1 nmol/min) was used as a selective ET_B receptor antagonist[24] (American Peptide Co). This dose has been shown to completely inhibit venoconstriction to the selective ET_B receptor agonist sarafotoxin S6c.[25]

The endogenous NO system in the forearm was inhibited by use of an "NO clamp," as described previously.[26] The NO synthase inhibitor L-N^G monomethyl-arginine (L-NMMA; Institut fur Pharmazie, Universitat Leipzig) was continuously infused at a rate of 200 μg/100 mL forearm volume per minute to achieve maximal inhibition of local NO synthase.[27–29] Sodium nitroprusside (SNP), an exogenous NO donor (Merck) was then coinfused at titrated doses (12 to 30 ng/min). After 8 minutes of L-NMMA infusion, when steady state forearm blood flow was obtained, SNP was coinfused in incremental doses and titrated until baseline forearm blood flow had been restored. L-NMMA and SNP were then coinfused, at these rates, for the remainder of the study. This allowed simulation of normal basal NO activity during continuous inhibition of endogenous NO synthesis.

Aspirin (600 mg calcium acetylsalicylic acid; Carbasalatum Calcium, Dagra Pharma BV) was administered orally 30 minutes before measurements in one phase of study 1. Aspirin irreversibly inhibits cyclooxygenase (EC 1.14.99.1), which is responsible for the production of prostaglandins and thromboxanes. When given at a dose of 600 mg, aspirin inhibits bradykinin-stimulated endothelial production of prostacylin by at least 85% with recovery occurring over the next 6 hours.[30]

Measurements

Forearm Blood Flow

Forearm blood flow was measured simultaneously in both arms by venous occlusion plethysmography using calibrated mercury-in-Silastic strain-gauges applied to the widest part of the forearm.[3,27,31] The hands were excluded from the circulation during each measurement period by inflation of a wrist cuff to 220 mm Hg. Upper arm cuffs were intermittently inflated to 40 mm Hg for 10 seconds every 15 seconds to temporarily prevent venous outflow from the forearm and thus obtain plethysmographic recordings. Recordings of forearm blood flow were made over 2.5-minute periods at 5-minute intervals (study 1) and over 3-minute periods at 10-minute intervals (study 2). Venous occlusion plethysmography was performed using a dual-channel strain-gauge plethysmograph (Hokanson), and calibration was achieved using the internal standard of the Hokanson plethysmography unit. In study 1, a microcomputer-based R-wave–triggered system for online semicontinuous monitoring was used,[32] whereas in study 2, voltage output was transferred to a Macintosh personal computer (Classic II; Apple Computer) using a MacLab analog-digital converter and Chart software (version 3.2.8; both from AD Instruments).

Blood Pressure

Blood pressure was monitored during each study using either continuous intra-arterial measurements in the infused arm (study 1) or a semiautomated noninvasive oscillometric method in the noninfused arm (study 2).[33] Blood pressure in study 2 was measured immediately after each forearm blood flow measurement, thereby avoiding any effect of venous congestion caused by this procedure on blood flow.

General Study Design

Subjects rested recumbent throughout each study with both forearms resting slightly above the level of the heart. Strain gauges and arm cuffs were applied, and the left brachial artery cannula was sited. Before the administration of drugs, saline was infused for at least 30 minutes,

Baseline Hemodynamic Values During Saline Infusion Before Infusion of Drugs

Parameter	Study 1			Study 2		
	BQ-123 (100 nmol/min)	BQ-123+ NO-Clamp	BQ-123+ Aspirin	BQ-123 (10 nmol/min)	BQ-123+ BQ-788	BQ-788 (1 nmol/min)
Forearm blood flow (mL/100 mL per minute)						
Infused arm	3.7±0.4	3.7±0.3	4.1±0.5	3.6±0.6	4.5±1.0	4.5±0.7
Control arm	4.6±0.5	3.4±0.2	3.4±0.5	3.4±0.6	3.3±0.8	3.1±0.4
Mean arterial pressure, mm Hg	78±2	82±3	79±2	83±5	90±3	93±4

Forearm blood flow in infused and control arm and mean arterial pressure in each phase of studies 1 and 2 at baseline before infusion of, respectively, BQ-123; BQ-123 SNP, and L-NMMA; BQ-123; BQ-123, BQ-123 and BQ-788; and BQ-788. There were no significant differences in baseline forearm blood flow or mean arterial pressure between the phases of each study.

n=8 in each phase of studies 1 and 2.

during which baseline measurements of forearm blood flow were made.

Study 1: Inhibition of NO Synthase and Prostanoid Generation With ET_A Receptor Blockade

Eight subjects were studied on three separate occasions, each separated by at least 1 week. After baseline infusion of saline, BQ-123 was infused for 90 minutes: on one occasion, during saline coinfusion; on another, after stabilization of the NO-clamp; and on another, after systemic inhibition of prostanoid generation. The effects of the NO-clamp on forearm blood flow were studied during a 2-hour period in 3 subjects (time control NO-clamp).

Study 2: Separate and Combined Blockade of ET_A and ET_B Receptors

On 2 separate study days, in 8 subjects, the ET_A receptor antagonist BQ-123 was infused for 120 minutes alone or during coinfusion of BQ-788, also for 120 minutes. On a separate occasion, BQ-788 was infused alone for 120 minutes in 8 subjects (2 of whom also participated in the earlier parts of study 2).

Analysis

Blood flow in both forearms was obtained from the mean of the last five consecutive recordings of each measurement period. Because wrist cuff inflation results in a transient forearm vasoconstriction, recordings made in the first 60 seconds after wrist cuff inflation were not used for analysis. The ratio of flows in the infused and noninfused arms was calculated for each time point and expressed as percentage change from baseline or, in the NO-clamp experiments, as percentage change from the average of the last four recordings during NO-clamping, before the administration of BQ-123. In both studies, plethysmographic data listings were extracted from data files, and forearm blood flows were calculated for individual venous occlusion cuff inflations using a template spreadsheet (Excel 5.0; Microsoft). All results are expressed as mean±SEM. Data were examined by repeated measures ANOVA (study 1, SigmaStat; Jandel Corp; study 2, Excel 5.0; Microsoft). Statistical significance was taken at the 5% level.

Results

There were no significant changes in baseline hemodynamics between phases of each study (Table) and no change in blood pressure or blood flow in the noninfused forearm during the course of the studies.

Study 1

Baseline forearm blood flow was restored during the NO-clamp (basal infused forearm blood flow, 3.7±0.3; during basal NO clamp; 3.4±0.2; $P=.15$) and kept stable for at least 40 minutes before BQ-123 infusion was started. Blood flow in the infused forearm in the time control NO clamp protocol varied by <5% between baseline (pre–NO clamp) and with 120 minutes of NO clamping in 3 subjects.

BQ-123 caused progressive vasodilatation during coinfusion of saline and after inhibition of prostanoid generation ($P<.01$ for both). The response appeared to plateau at 60 minutes, and no differences were observed in these responses (38±9% versus 42±7% at 90 minutes; $P=.5$). The vasodilator response to BQ-123 was markedly reduced during NO-clamping (2±5% at 90 minutes, $P=.006$ versus saline coinfusion) (Fig 1).

Study 2

Both BQ-123 alone and coadministration of BQ-123 and BQ-788 caused progressive vasodilatation ($P<.001$) that appeared to plateau at 60 minutes (Fig 2). The vasodilatation to BQ-123 alone was significantly greater than that during coinfusion with BQ-788 (76±13% versus 47±14% at 120 minutes, $P<.001$). BQ-788 alone caused a small but consistent reduction in forearm blood flow (20±3% at 120 minutes, $P<.001$) (Fig 2).

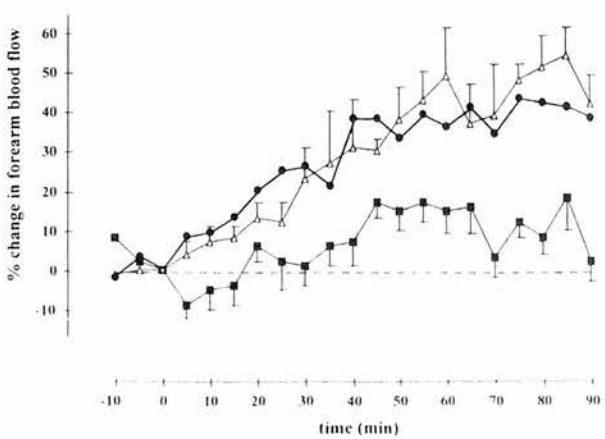

Figure 1. Eight subjects received brachial artery infusion of BQ-123 (100 nmol/min) during coinfusion of saline (●), BQ-123 (100 nmol/min) during inhibition of prostanoid generation (△), or BQ-123 (100 nmol/min) during inhibition of NO generation (■). Slow-onset vasodilatation occurred in response to BQ-123; this response was attenuated during NO clamp but not during inhibition of prostanoid generation.

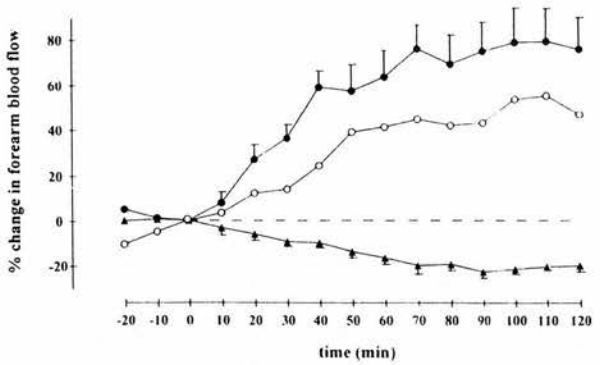

Figure 2. Eight subjects received brachial artery infusion of BQ-123 (10 nmol/min) alone (●), BQ-788 (1 nmol/min) alone (▲), or BQ-123 (10 nmol/min) coinfused with BQ-788 (1 nmol/min) (○). Slow-onset vasodilatation occurred in response to BQ-123; this response was attenuated during coinfusion of BQ-788. BQ-788 infusion alone caused a small but significant vasoconstriction.

Discussion

In two centers, we have demonstrated slow-onset forearm vasodilatation in response to local arterial infusion of the selective ET_A receptor antagonist BQ-123, confirming the importance of endogenous ET-1 in the mediation of vascular tone. From these data, it appears that this vasodilator response is caused in large part by increased generation of NO, which could be mediated by stimulation of the endothelial ET_B receptor. Indeed, our observation that the vasodilator response to combined ET_A and ET_B receptor antagonism was significantly less than that to selective ET_A receptor antagonism alone probably reflects the presence of an endogenous ET_B-mediated vasodilator tone. This is further supported by the local vasoconstrictor effect of ET_B receptor antagonism in the forearm resistance vessels.

In the present study, to exclude the influence of the endogenous NO system in mediation or modulation of the effects of ET-1, L-NMMA was infused to inhibit endogenous local generation of NO. SNP was coinfused with L-NMMA to restore baseline blood flow[26] because local inhibition of NO would otherwise result in vasoconstriction. In this situation, endogenous NO is replaced with exogenous NO, in effect applying a clamp to the local endogenous NO system. Using this technique we have shown, for the first time in humans in vivo, that the vasodilatation to BQ-123 is in large part related to NO generation. Inhibition of endogenous prostanoid generation by oral administration of aspirin has no effect on basal forearm blood flow or systemic hemodynamics and, more importantly, had no effect on the response to BQ-123, indicating that the dilator prostanoids do not provide an important contribution to the vasodilator response to BQ-123. Almost all of the response to BQ-123 appeared to be blocked by NO clamping. However, on the basis of vasodilatation to the ECE inhibitor phosphoramidon[31] in previous studies,[5] we think it is likely that at least part of the response to BQ-123 is directly due to withdrawal of endogenous ET_A-mediated vasoconstriction.

Selective ET_A antagonism inhibits the actions of ET-1 at the ET_A receptor while allowing its actions at the ET_B receptor to be unopposed. ET-1 can stimulate both the endothelial ET_B receptor to cause dilatation and the vascular smooth muscle ET_B receptor to cause vasoconstriction. Therefore, the overall effect depends on a balance between these two actions. Unfortunately, there are no available pharmacological tools that have been shown clearly to distinguish between the endothelial and vascular smooth muscle ET_B receptors. We have shown that coinfusion of the ET_B receptor antagonist BQ-788 reduces the vasodilator response to BQ-123, suggesting that the balance of effects of ET-1 favors vasodilatation via the endothelial ET_B receptor. This is further supported by the vasoconstriction in these vessels to BQ-788 alone and by the lesser degree of vasodilatation to the combined ET_A/ET_B endothelin receptor antagonist TAK-044[4] than to the ET_A-selective agent BQ-123.[3] It is possible that the predominant effects of intra-luminal infusion of BQ-788 selectively affect the endothelial ET_B receptor because the drug has better access to the endothelial than to the smooth muscle receptors. However, we believe this is unlikely because ET-1 and BQ-123 find ready access to the smooth muscle. The response to BQ-788 may indicate either displacement of ET-1 from, or failure of clearance of ET-1 by, ET_B receptors.[35] However, our present results cannot distinguish between these effects.

The observation that selective ET_A receptor blockade not only antagonizes direct ET_A receptor–mediated constriction but also preserves beneficial ET_B receptor–mediated vasodilator tone and enhances endogenous NO generation may have important implications in the use of endothelin antagonists as treatments in cardiovascular disease. For example, the increased NO generation caused by ET_A receptor antagonists is potentially beneficial in ischemic heart disease. However, the clinical relevance of our findings in various pathophysiological conditions cannot be fully determined from the present study because endothelin receptors may be modified under these circumstances. Indeed, in ischemic heart disease, there appears to be upregulation of human coronary ET_B receptors,[36] and this is associated, in heart failure, with enhanced vasoconstrictor responses to sarafotoxin S6c in both the forearm[37] and coronary circulation,[38] whereas the response to BQ-788 appeared similar to that of controls.[39] Clearly, at some stage, it will be necessary to examine the integrated physiology of systemic ET_A and ET_B blockade in physiological and pathophysiological conditions to fully understand the relative importance of the receptor subtypes.

In summary, we have demonstrated that the local vasodilator response to selective ET_A receptor antagonism in human forearm resistance vessels is derived in large part from increased NO-mediated vasodilatation, most probably mediated by the endothelial ET_B receptor. Although our observations were made in the forearm resistance vessels, these vessels are generally representative of other vascular beds[40,41] and, importantly, reflect the interaction of these systems in vivo. Our results may indicate new therapeutic uses for ET_A receptor antagonists because increased NO synthesis may be a desirable effect in, for example, ischemic heart disease. One could also postulate that enhanced endogenous NO generation may be responsible for the headaches that are a recognized side effect of ET receptor antagonists.

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Endogenous angiotensin II does not contribute to sympathetic venoconstriction in dorsal hand veins of healthy humans

Background: **Sympathetically mediated venoconstriction is augmented by exogenously administered angiotensin II. This study was designed to assess whether endogenous angiotensin II influences sympathetically mediated venous tone.**

Methods: **Responses of dorsal hand veins to local intravenous administration of subsystemic doses of losartan, an angiotensin II type-1 receptor antagonist, were assessed with use of a well-validated displacement technique in eight healthy male volunteers. In a four-phase study, responses to local infusions of angiotensin II (4 to 64 ng/min) and norepinephrine (1 to 128 ng/min) or to sympathetic venoconstriction produced by a single deep breath were compared in the presence of either saline placebo or 30 μg/min losartan. Each phase of the study was conducted on a separate day, in random order, and each phase was separated by at least 1 week.**

Results: **Angiotensin II ($p = 0.03$) and norepinephrine ($p < 0.001$) caused dose-dependent venoconstriction. Losartan attenuated the venoconstriction induced by angiotensin II ($p = 0.048$) but had no effect on the responses to norepinephrine or the venoconstriction induced by a single deep breath.**

Conclusions: **In contrast to exogenously administered angiotensin II, basal endogenous angiotensin II does not influence sympathetically mediated venoconstriction in healthy humans. However, endogenous angiotensin II may have a role in circumstances of renin-angiotensin system activation, such as salt depletion. (Clin Pharmacol Ther 1997;62:327-33.)**

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The renin-angiotensin system plays a key role in the regulation of circulating blood volume and body sodium balance[1] through the generation of the potent vasoconstrictor peptide angiotensin II. In circumstances of volume depletion, angiotensin II sustains blood pressure through elevation of peripheral vascular resistance and reduction of central venous capacitance. These vasoconstrictor and venoconstrictor effects are mediated by the vascular smooth muscle angiotensin II type-1 (AT_1) receptor. In addition to producing vasoconstriction directly, angiotensin II potentiates the activity of the sympathetic nervous system. Administration of exogenous angiotensin II at doses insufficient to cause vasoconstriction directly augments sympathetically mediated constriction of dorsal hand veins[2] and forearm resistance vessels[3] of healthy subjects by a prejunctional effect.

Previous local vein studies that have assessed the influence of the renin-angiotensin system in veins have used angiotensin converting enzyme (ACE) inhibitors.[4-6] However, local ACE inhibition will potentially inhibit both the generation of angiotensin II and the metabolism of bradykinin and is therefore nonspecific in its action. Moreover, if locally active, the drugs will not affect the circulating plasma concentrations of angiotensin II to which the vessels are

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exposed. Losartan is a selective AT_1-receptor antagonist that has recently become available for clinical use. In contrast to ACE inhibitors, AT_1-receptor antagonists are both specific and capable of directly blocking the responses to angiotensin II. Previously, we have shown that locally infused losartan inhibits the vasoconstriction induced by angiotensin II in human forearm resistance vessels.[7]

Capacitance vessels influence cardiac output and, consequently, other hemodynamic parameters through the regulation of cardiac preload. However, when in vivo vascular responses in humans are examined, systemic drug administration can cause concomitant effects on many other tissues, as well as influence neurohumoral reflexes through changes in systemic hemodynamics. Therefore vascular responses cannot be wholly attributed to a direct effect of the drug.[8] However, the assessment of venous compliance in a single dorsal hand vein with use of the Aellig displacement technique[9,10] permits the direct effect of locally active subsystemic doses of infused drugs to be examined without these confounding influences.[8] Moreover, the cutaneous limb veins, in contrast to the skeletal muscle veins, participate in the sympathetic venomotor reflexes.[2,11] Thus responses in hand veins are representative of the physiologic regulation of central venous capacitance and cardiac preload.[12]

The first aim of this study was to define a locally active dose of losartan that would selectively and effectively inhibit angiotensin II–mediated venoconstriction. The second aim was to evaluate whether basal endogenous angiotensin II contributes to sympathetically mediated venoconstriction through an action at the AT_1-receptor.

METHODS

Subjects. Eight healthy male subjects between 24 and 36 years old participated in four studies that were undertaken in accordance with the Declaration of Helsinki (1989) of the World Medical Association and with the approval of the Lothian Research Ethics Committee. The written informed consent of each subject was obtained before the study. None of the subjects received vasoactive or nonsteroidal anti-inflammatory drugs in the week before each phase of the study, and all abstained from alcohol for 24 hours and from food and caffeine-containing drinks for at least 5 hours before each study. All subjects were nonsmokers. Studies were performed in a quiet temperature-controlled room maintained at 23.5° to 24.5° C.

Drugs. Losartan (DuPont-Merck Inc., Wilmington, Del.), norepinephrine (Levophed; Sanofi Winthrop Ltd., Guildford, England), and angiotensin II (Clinalfa AG, Läufelfingen, Switzerland) were dissolved in physiologic saline (0.9%; Baxter Healthcare Ltd., Thetford, England) and administered into a dorsal hand vein. To prevent its oxidation, norepinephrine was dissolved in saline solution that contained 0.1% ascorbic acid (Evans Medical Ltd., Langhurst, England). The dose of losartan used (30 μg/min) was chosen to achieve an effective subsystemic and locally active concentration and was based on earlier pilot and published studies.[7,13] Locally administered losartan at 30 μg/min is sufficient to cause a 37-fold increase in the dose of angiotensin II required to produce a 20% vasoconstriction in forearm resistance vessels.[7]

Intravenous administration. A 23-gauge butterfly cannula (Abbott Ireland, Sligo, Ireland) was inserted into the dorsal hand vein in the direction of flow and attached to a 16-gauge epidural catheter (Portex Ltd., Hythe, England). Patency was maintained by infusion of saline through an IVAC P1000 syringe pump (IVAC Ltd., Basingstoke, England). The total rate of intravenous infusions was maintained constant throughout all studies at 0.25 ml/min, and the same hand vein was used for each study.

Dorsal hand vein size. The left hand was supported above the level of the heart by means of an arm rest. The internal diameter of the dorsal hand vein, distended by inflation of an upper arm cuff to 30 mm Hg, was measured by the technique of Aellig[9,10,14] with use of a linear variable differential transducer (LVDT; model 025 MHR, Lucas Schaevitz Inc., Slough, Berkshire, England). In brief, the LVDT was mounted on the dorsum of the hand with a small tripod, and a magnetized rod was passed through the core to rest on the summit of the vein approximately 1 cm proximal to the tip of the infusion cannula. Vertical displacement of the rod causes a linear change in the voltage generated by the LVDT, reflecting changes in the internal diameter of the vein. Absolute changes in vein size were determined by calibration with standard displacements.

The deep breath stimulus was performed as described previously.[2,14] In brief, when vein size was stable, subjects were asked to breathe out fully before breathing in as deeply as possible. They were asked to hold this inspiration for 10 seconds and to avoid any tendency to breathe out. The technique

was practiced before the study to ensure that subjects did not perform a Valsalva maneuver.

Blood pressure was monitored in the noninfused arm at intervals throughout each study using a semiautomated noninvasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc., Tokyo, Japan).[15]

Study design. This was a four-phase, single-blind randomized study. Subjects rested semirecumbent throughout each study, and the dorsal hand vein was cannulated and the LVDT sited. Saline solution was infused for the first 30 minutes and vein size was measured every 5 minutes before subjects participated in one of the following protocols:

- *Protocol 1—Effect of losartan on responses to angiotensin II and norepinephrine:* Six subjects attended each of 2 study days, at least 1 week apart, and received infusion of saline placebo or losartan at 30 μg/min for 120 minutes, on separate occasions, in random order. After a 10-minute infusion of saline solution or losartan, angiotensin II was coinfused at 4 and 64 ng/min for 7 minutes at each dose.[16] Vein size was measured at the end of each infusion period. After a 15-minute saline infusion, norepinephrine was coinfused at incremental doubling doses from 1 to 128 ng/min[16] for 10 minutes at each dose. Vein size was measured every 5 minutes during saline infusion and at the end of the infusion of each dose of norepinephrine.
- *Protocol 2—Effect of losartan on venoconstriction to a single deep breath:* Six subjects attended each of 2 study days, at least 1 week apart, and received infusion of saline placebo or losartan at 30 μg/min for 60 minutes, on separate occasions, in random order. Venoconstriction to a single deep breath was measured 20 and 10 minutes before infusion of losartan or saline solution, and the mean was taken as the baseline response. The single deep breath response was repeated at 20, 40, and 60 minutes during infusion of losartan or saline solution. Vein size was measured every 5 minutes throughout the study.

Four subjects participated in both protocols.

Data analysis and statistics. Basal vein size was calculated by taking the mean of the last three measurements before receipt of the randomized allocation of losartan or saline solution. Because absolute basal vein size can vary greatly between subjects, responses to angiotensin II, norepinephrine, and a deep breath are expressed as the percentage change in vein size from basal. All results are expressed as mean values ± standard error of the mean.

Data were examined by one- and two-way ANOVA with repeated measures, and the Student *t* test and distribution when appropriate, with Excel 4.0 (Microsoft Corp., Redmond, Wash.). Statistical significance was taken at the 5% level. Confidence intervals were obtained for the mean and area under the curve (AUC) of the single deep breath responses over 60 minutes of saline and losartan infusion. On the basis of the individual single deep breath responses, the 90% power to detect a difference between saline and losartan responses was calculated at a significance level of 5%.

RESULTS

There were no significant differences in blood pressure or heart rate within and between the study periods (Table I). Baseline vein sizes were similar between study periods, with no significant differences between the 4 study days.

- *Protocol 1—Effect of losartan on venoconstriction to angiotensin II and norepinephrine:* Locally infused angiotensin II ($p = 0.03$; Fig. 1) and norepinephrine ($p < 0.001$; Fig. 2) caused dose-dependent venoconstriction of the infused superficial hand veins. Venoconstriction to angiotensin II ($p = 0.048$ versus saline solution; Fig. 1) but not norepinephrine ($p = 0.48$; Fig. 2) was inhibited by coinfusion of losartan.
- *Protocol 2—Effect of losartan on venoconstriction to a single deep breath:* Basal vein diameters were unaffected by infusion of losartan at 30 μg/min for 60 minutes. A single deep breath caused a −30% reduction in vein diameter. There were no significant differences in the venoconstriction to a single deep breath when performed in the presence of losartan or saline infusion (Fig. 3), with an overall mean venoconstriction of 24.9% (95% confidence intervals [CI], 21.5% to 28.3%) and 24.5% (95% CI, 22.2% to 26.7%), respectively. From these responses, the study has a 90% power of detecting an 8.1% difference at each time point. The difference between the AUC of the saline and losartan responses was −1.4% (95% CI, −10.5% to +7.8%). Venoconstriction to a single deep breath tended to diminish with time, although this effect was not statistically significant ($p = 0.71$; ANOVA).

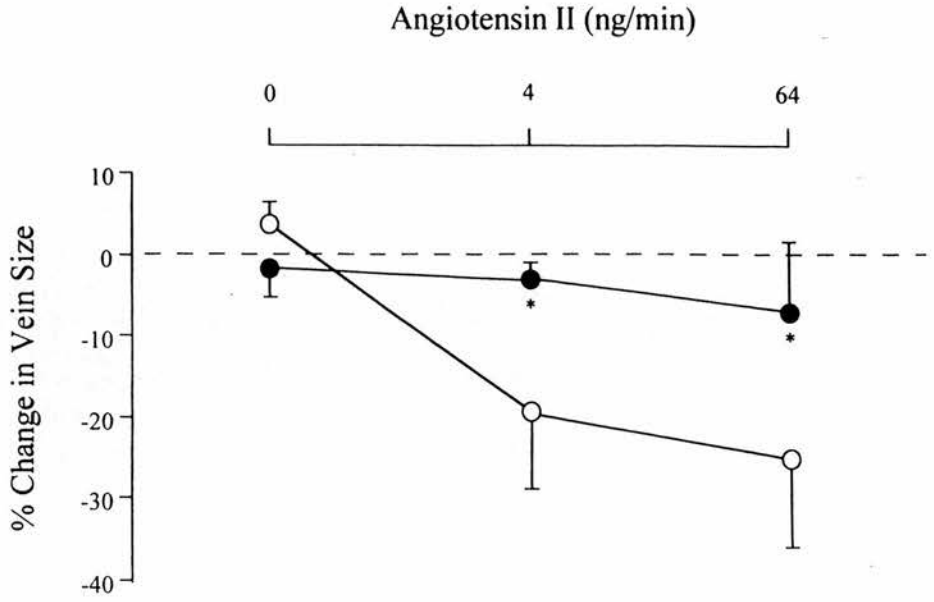

Fig. 1. Effects of losartan on venoconstriction induced by angiotensin II. Angiotensin II coinfused with saline *(open circles)* or 30 μg/min losartan *(solid circles)*. *p = 0.048 versus saline solution; ANOVA.

Table I. Hemodynamic characteristics and vein sizes on the 4 study days

	Protocol 1				*Protocol 2*			
	Saline solution		*Losartan*		*Saline solution*		*Losartan*	
	Basal	*Final*	*Basal*	*Final*	*Basal*	*Final*	*Basal*	*Final*
Blood pressure (mm Hg)								
Systolic	118 ± 5	119 ± 7	117 ± 7	116 ± 4	116 ± 5	111 ± 4	123 ± 9	111 ± 3
Diastolic	71 ± 3	71 ± 2	76 ± 4	72 ± 3	69 ± 3	68 ± 2	67 ± 1	67 ± 2
Mean	87 ± 4	87 ± 5	89 ± 6	87 ± 3	85 ± 4	82 ± 3	86 ± 5	82 ± 3
Heart rate (beats/min)	59 ± 4	58 ± 3	64 ± 3	62 ± 3	66 ± 4	57 ± 3	63 ± 4	60 ± 2
Vein size (mm)	1.1 ± 0.2	0.2 ± 0.1	1.2 ± 0.1	0.1 ± 0.1	1.1 ± 0.2	1.1 ± 0.2	0.8 ± 0.1	0.9 ± 0.1

Data are mean values ± SEM.

DISCUSSION

In agreement with previous studies using angiotensin II[4-6] and norepinephrine[6,13,16] in the dorsal hand veins, locally active subsystemic infusions of both agents caused dose-dependent venoconstriction. Losartan at a dose of 30 μg/min, in keeping with its pharmacologic actions as an AT_1-receptor antagonist, inhibited the venoconstriction to exogenous angiotensin II but had no effect on the venoconstriction caused by the control venoconstrictor, norepinephrine. There was no effect of losartan on basal vein size because these vessels are fully relaxed in the basal state.[8-10] Systemic doses of losartan can also inhibit venoconstriction to angiotensin II,[17] but here the response reflects the combined activity of losartan and its long-acting active metabolite, E-3174, and the effects of other influences associated with systemic AT_1-receptor antagonism.

The potential interaction between the sympathetic nervous and renin-angiotensin systems has been extensively reported. In vitro studies have shown that responses to sympathetic nerve stimulation are enhanced by angiotensin II in both animals and humans.[18,19] This effect occurs predominantly through the prejunctional release of norepinephrine.[18] Evidence supporting the physiologic role of

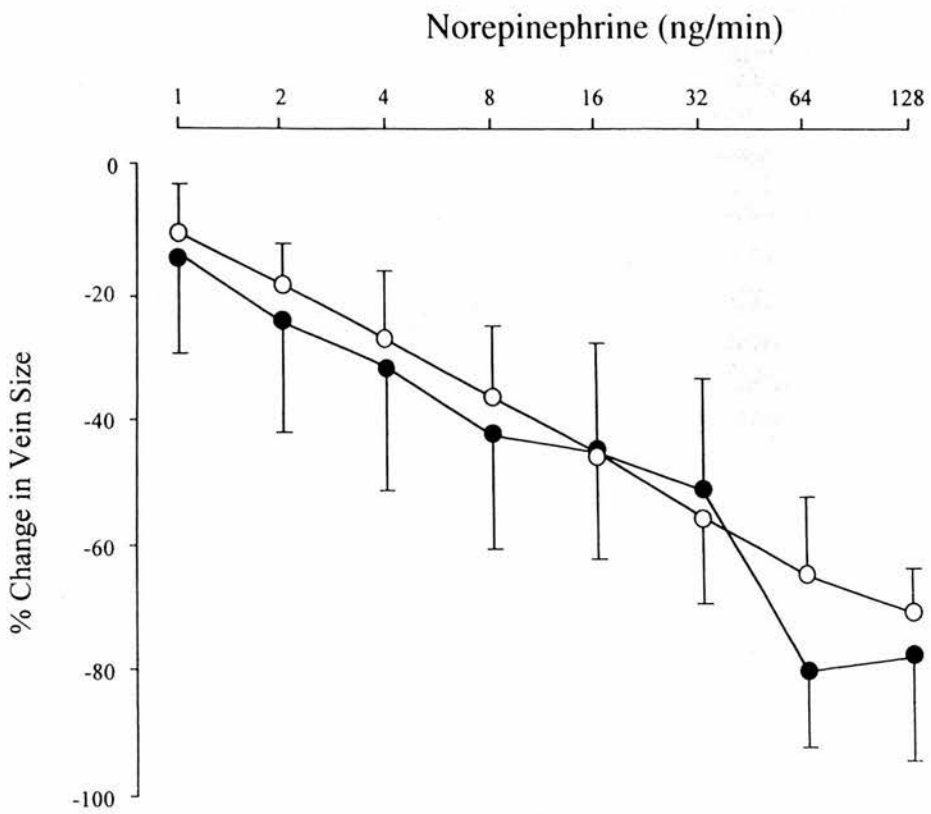

Fig. 2. Effects of losartan on dose-response curves for norepinephrine. Norepinephrine was coinfused with saline solution *(open circles)* or 30 μg/min losartan *(solid circles)*. There were no significant differences ($p = 0.48$) between responses during saline and losartan infusions.

this interaction in vivo in humans with use of the dorsal hand vein technique has been reported.[2] Locally infused angiotensin II, at doses insufficient to cause venoconstriction directly, enhances sympathetically stimulated venoconstriction produced by a single deep breath. If endogenous tissue or circulating angiotensin II[18] maintains normal sympathetically mediated venoconstriction, then losartan should reduce the response to a single deep breath. However, we have found no attenuation of the single deep breath response with losartan. The 95% confidence intervals indicate that if angiotensin II provides any contribution to sympathetically mediated venoconstriction, it is rather small. In addition, this lack of overall effect cannot reflect a combination of reduced prejunctional norepinephrine release and increased postjunctional α-adrenergic receptor sensitivity because venoconstriction to norepinephrine was unaffected by losartan. This suggests that, under physiologic conditions, endogenous angiotensin II either has little or no influence on sympathetically stimulated venous tone or, less likely, its influence is through a mechanism that is not mediated by an AT_1-receptor. There was a trend for the deep breath response to decrease with time, consistent with previous studies that used repeated single deep breath responses.[14,20] This effect was present during both saline and losartan infusions and may represent a physiologic waning of the response (i.e., tachyphylaxis).

Unlike resistance vessels, dorsal hand veins have very little resting tone and the effects of venodilators are small and variable.[8-10] Therefore hand veins are generally preconstricted before assessment of responses to venodilators. The absence of a venodilatory effect of losartan at rest, during sympathetic stimulation, and during norepinephrine infusion would suggest that endogenous angiotensin II does not contribute to venous tone under physiologic circumstances. By administering losartan through the brachial artery, we[7] and others[13] have shown that angiotensin II, in healthy sodium-replete sub-

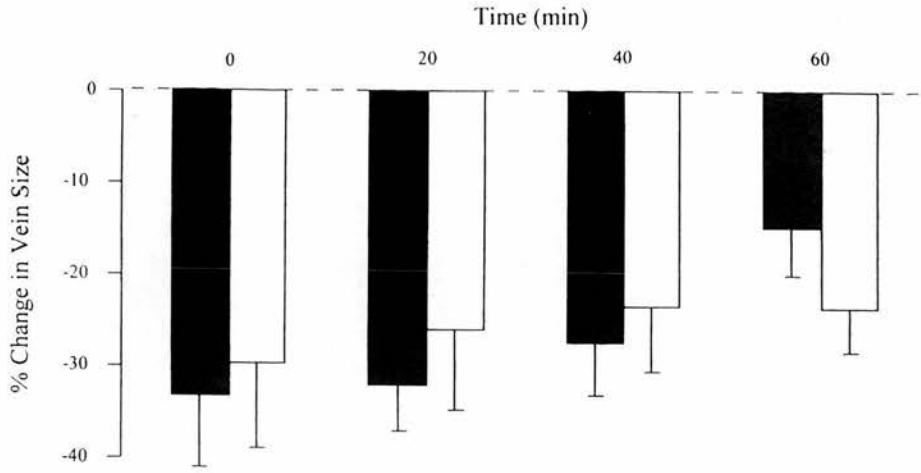

Fig. 3. Effects of losartan on sympathetically mediated venoconstriction. Venoconstriction resulting from single deep breath during infusion of saline solution *(open squares)* or 30 μg/min losartan *(solid squares)*. There were no significant differences ($p = 0.80$) between responses during saline and losartan infusions.

jects on a Western diet, does not contribute to forearm resistance vessel tone, suggesting that physiologic concentrations of angiotensin II are not active in either peripheral resistance or capacitance vessels. Although angiotensin II clearly does contribute to vascular tone under conditions of renin-angiotensin system activation, such as sodium depletion or chronic heart failure, the mechanism by which losartan and E3174 reduce blood pressure in healthy sodium-replete humans is unlikely to be peripheral venodilation or vasodilation. This does not preclude the possibility that a hypotensive effect of angiotensin II antagonism might be mediated through actions on the kidney[21,22] or central nervous system.[23,24] Interestingly, the evidence for a hypotensive action of losartan in sodium-replete humans is contradictory.[25,26] Indeed, in the main study,[25] which showed a hypotensive action of losartan, subjects had an elevated basal plasma renin activity, suggesting that they had an activated renin-angiotensin system, presumably secondary to mild sodium depletion. Further studies are required to show whether endogenous angiotensin II contributes to basal venous tone and sympathetic venoconstriction under circumstances of renin-angiotensin system activation.

In contrast to earlier findings,[4,5] Zarnke and Feldman[27] reported a significant venodilation to ACE inhibition in small or preconstricted dorsal hand veins. However, we were unable to demonstrate any significant venodilation to losartan in veins under basal conditions or during norepinephrine venoconstriction. Our observations would therefore suggest that this venodilation to ACE inhibition is not mediated through a reduction in angiotensin II generation but may result from the accumulation of vasodilator peptides such as bradykinin or substance P. Alternatively, the findings of Zarnke and Feldman[27] may represent a difference in responses between larger and smaller hand veins or a nonspecific vasodilatory effect of high drug concentrations in small low-flow veins.

In summary, when losartan is given at a locally active and subsystemic dose of 30 μg/min into the dorsal hand vein, it inhibits the venoconstriction to angiotensin II but not norepinephrine. Losartan does not appear to have an effect on resting or sympathetically stimulated venous tone. Therefore it would appear that endogenous angiotensin II does not contribute to the sympathetic venoconstrictor reflex in hand veins of healthy humans. However, endogenous angiotensin II may have a role in circumstances of renin-angiotensin system activation, such as salt depletion.

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Endothelin ET_A and ET_B Receptors Cause Vasoconstriction of Human Resistance and Capacitance Vessels In Vivo

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Background The role of endothelin ET_B receptors in mediating vasoconstriction in humans is unclear. As yet, there have been no in vivo studies in resistance vessels, and in vitro data have been contradictory. We therefore investigated the function of ET_B receptors in vivo in human forearm resistance and hand capacitance vessels using endothelin-1 as a nonselective agonist at ET_A and ET_B receptors and endothelin-3 and sarafotoxin S6c as selective agonists at the ET_B receptor.

Methods and Results A series of single-blind studies were performed, each in six healthy men. Brachial artery infusion of endothelin-1 and endothelin-3 caused slow-onset dose-dependent forearm vasoconstriction. Although endothelin-3 caused significantly less forearm vasoconstriction than endothelin-1 at low doses, vasoconstriction was similar to the two isopeptides at the highest dose (60 pmol/min). Endothelin-3 caused transient forearm vasodilatation at this dose, whereas endothelin-1 showed only a nonsignificant trend toward causing early vasodilatation. Intra-arterial sarafotoxin S6c caused a progressive reduction in forearm blood flow, although less than that to endothelin-1 ($P=.04$). Dorsal hand vein infusion of sarafotoxin S6c caused local venoconstriction that was also less than that to endothelin-1 ($P=.002$).

Conclusions Selective ET_B receptor agonists cause constriction of forearm resistance and hand capacitance vessels in vivo in humans, suggesting that both ET_A and ET_B receptors mediate vasoconstriction. Hence, antagonists at both ET_A and ET_B receptors, or inhibitors of the generation of endothelin-1, may be necessary to completely prevent vasoconstriction to endogenously generated endothelin-1. (***Circulation.*** **1995;92:357-363.**)

Key Words • endothelin • vasoconstriction • vessels

The endothelins are a family of 21-amino-acid peptides with potent and characteristically sustained vasoconstrictor and vasopressor actions.[1] Endothelin-1 is the predominant isopeptide generated by the vascular endothelium.[2] Endothelin-2 and endothelin-3 are more difficult to detect in humans and are probably less important in their cardiovascular effects.

Two specific receptors for the endothelins have been isolated by in vitro expression of cloned human cDNA.[3,4] The ET_A receptor has a high affinity for endothelin-1, with a K_i of 0.6 nmol/L for endothelin-1 compared with 140 nmol/L for endothelin-3.[5] ET_A receptor mRNA was initially reported to be highly expressed in human aorta but not cultured human endothelial cells, suggesting selective vascular expression of this receptor in smooth muscle cells.[3] The ET_B receptor has equal affinity for all three endothelins, with K_i values for endothelin-1 and endothelin-3 of 0.12 and 0.06 nmol/L, respectively.[5] The ET_B receptor has been reported to be highly expressed in cultured endothelial cells[4] but not vascular smooth muscle cells.[6]

On the basis of the greater vasoconstrictor potency of endothelin-1 than endothelin-3 and the apparently exclusive expression of ET_A receptors in vascular smooth muscle, vasoconstriction to endothelin-1 was initially thought to be mediated solely by vascular smooth muscle cell ET_A receptors. Vascular ET_B receptors located on endothelial cells were thought only to mediate generation of endothelium-derived dilator substances. More recent evidence suggests that ET_B receptor mRNA is expressed in human vascular smooth muscle obtained from the aorta, pulmonary artery, and coronary artery,[7] consistent with a potential vasoconstrictor role for this receptor. Indeed, in animals, there is functional evidence for ET_B receptor–mediated vasoconstriction in vitro, particularly in venous tissue.[8-13] In addition, selective ET_B receptor agonists have pressor effects in animals in vivo.[12,14-16] However, the contribution of ET_B receptors to vasoconstriction is variable and appears to depend markedly on species, vessel type, and vessel size.[17] Furthermore, the functional significance of such vascular smooth muscle ET_B receptors in humans is unclear, with in vitro studies reporting that ET_B receptors make either a minimal[11,17-24] or, at most, a moderate contribution[25-27] to vasoconstriction, depending on the types of vessel studied.

The relevance of this issue is emphasized by the recent development of both selective and nonselective antagonists at ET_A and ET_B receptors. For example, selective ET_A receptor antagonists will block vasoconstriction mediated by ET_A receptors but may not block all constriction to endothelin-1 if there are also vasoconstrictor ET_B receptors. However, if the putative constrictor ET_B receptor is relatively unimportant in humans, then blocking both ET_A and ET_B receptors may cause less vasodilatation than blocking the ET_A receptor alone, because such receptor antagonists will also block the endothelial dilator ET_B receptor.

In view of the inconsistent results with and the potential disadvantages of in vitro studies, we investigated the function of endothelin ET_A and ET_B receptors in blood vessels in vivo in humans. We used endothelin-1 as a nonselective agonist at ET_A and ET_B receptors and endothelin-3 and sarafotoxin S6c as selective ET_B receptor agonists; these peptides have about 2000- and 300 000-

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fold selectivity, respectively, for the ET_B over the ET_A receptor.[5,28] Using locally active doses of these agents, we assessed responses both of resistance vessels, using brachial artery administration,[29] and of capacitance vessels, using dorsal hand vein administration.[30-32] We used local doses of peptides so that interpretation of the results would not be confounded by direct effects of systemic administration on kidney, heart, or brain or by reflex effects consequent to changes in blood pressure.

Methods

Subjects

Twenty-four healthy male subjects between 22 and 38 years of age participated in these studies, which were conducted with the approval of the Lothian Medicine and Clinical Oncology Ethics of Medical Research Subcommittee and with the written informed consent of each subject. No subject received vasoactive or nonsteroidal anti-inflammatory drugs in the week before each phase of the study, and all abstained from alcohol for 24 hours and from food, caffeine-containing drinks, and cigarettes for at least 3 hours before any measurements were made. All studies were performed in a quiet room maintained at a constant temperature of between 22°C and 25°C.

Drugs

Pharmaceutical-grade endothelin-1 (Clinalfa, NovaBiochem), endothelin-3 (Clinalfa), and sarafotoxin S6c (Sigma Chemical Co Ltd) were administered. A single dose of each peptide was used in individual studies because the slow onset and long-lasting action of the endothelin isopeptides preclude the use of repeated doses in a single study to examine conventional dose-response relations.[33] The peptides were dissolved in physiological saline (0.9%; Baxter Healthcare Ltd).

Intra-arterial Administration

The left brachial artery was cannulated under local anesthesia (1% lignocaine; Astra Pharmaceuticals) with a 27-standard wire gauge steel needle (Coopers Needle Works) attached to a 16-gauge epidural catheter (Portex Ltd). Patency was maintained by infusion of 0.9% physiological saline via a Welmed P1000 syringe pump (Welmed Clinical Care Systems). The total rate of intra-arterial infusion was maintained constant throughout all intra-arterial studies at 1 mL/min.

Intravenous Administration

A vein on the dorsum of the left hand was cannulated in the direction of flow with a 23-gauge butterfly needle (Abbott) attached to a 16-gauge epidural catheter, without use of local anesthesia. The same vein was used in each subject for each individual study. Patency was maintained by infusion of 0.9% physiological saline via a Welmed P1000 syringe pump. The total rate of intravenous infusion was maintained constant throughout all studies at 0.25 mL/min.

Measurements

Forearm Blood Flow

Blood flow was measured in the infused and noninfused forearms by venous occlusion plethysmography[34] using indium/gallium-in-Silastic strain gauges[29] that were securely applied to the widest part of each forearm. The hands were excluded from the circulation during each measurement period by inflation of a wrist cuff to 220 mm Hg. Upper-arm cuffs were intermittently inflated to 40 mm Hg for 10 in every 15 seconds to temporarily prevent venous outflow from the forearm and thus obtain plethysmographic recordings. Recordings of forearm blood flow were made repeatedly over 3-minute periods unless otherwise stated. Voltage output from a dual-channel Vasculab SPG 16 strain-gauge plethysmograph (Medasonics Inc) was transferred to a Macintosh personal computer (Classic II, Apple Computer Inc) with a MacLab analog-to-digital converter and CHART software (v. 3.2.8; both from AD Instruments). Calibration was achieved by use of the internal standard of the Vasculab plethysmography unit.

Dorsal Hand Vein Diameter

The left hand was supported above the level of the heart by means of an arm rest. The ID of the dorsal hand vein, distended by inflation of an upper arm cuff to 30 mm Hg, was measured by the technique of Aellig.[30] In brief, a magnetized lightweight rod rested on the summit of the infused vein ~1 cm downstream from the tip of the infusion cannula. This rod passed through the core of a linear variable differential transformer (LVDT; model 025 MHR, Lucas Schaevitz Inc) supported above the hand by a small tripod, the legs of which rested on areas of the dorsum of the hand free of veins. If venoconstriction occurred while this cuff was inflated or if the cuff was deflated with consequent emptying of the vein, there was a downward displacement of the lightweight rod that caused a linear change in the voltage generated by the LVDT. The voltage output from the LVDT was transferred to a Macintosh personal computer by use of a MacLab analog-to-digital converter and CHART software. Standard displacements were used to calibrate the LVDT to determine the ID of the vein.

Blood Pressure

A well-validated semiautomated noninvasive oscillometric sphygmomanometer (Takeda UA 751, Takeda Medical Inc) was used to make duplicate measurements of blood pressure in the noninfused arm.[35]

Study Design

Four single-blind studies were performed, with the experimental subjects but not the investigators blinded to the peptide and dose administered in each study.

Forearm Resistance Bed Protocols

Subjects rested recumbent throughout each study. Strain gauges and arm cuffs were applied, and the left brachial artery cannula was sited. Saline was infused for 30 minutes, during which two measurements of forearm blood flow were made (at −20 and −10 minutes). Blood pressure was measured immediately after each forearm blood flow measurement, thereby avoiding any effect on forearm blood flow measurements of the venous congestion caused by this procedure.[36] Three protocols were then followed, each in separate groups of subjects, as follows.

Protocol 1: Low-dose intra-arterial endothelin-1 and endothelin-3. On four separate occasions, in random order, six subjects received brachial artery infusion of endothelin-1 and endothelin-3 at 1 and 5 pmol/min, each for 60 minutes. The choice of doses was based on previous work showing, in vivo, that 5 pmol/min of endothelin-1 causes slow-onset vasoconstriction in human forearm resistance vessels, reducing blood flow by ~40%.[29,33] Forearm blood flow was recorded from 3 minutes before to 5 minutes after the endothelin infusion was begun. Thereafter, measurements were made at 5-minute intervals for 60 minutes. Blood pressure was measured 60 minutes after the infusion was begun.

Protocol 2: High-dose intra-arterial endothelin-1 and endothelin-3. On two separate occasions, in random, balanced order, six subjects received endothelin-1 and endothelin-3 via the brachial artery at 60 pmol/min for 5 minutes, followed by physiological saline for 55 minutes. Because no significant vasodilatation had been observed in previous studies using intra-arterial endothelin-1 at 5 pmol/min,[29,33] we chose a dose of 60 pmol/min with the intention of stimulating sufficient endothelial generation of dilator substances to cause vasodilatation before the development of vasoconstriction. Forearm blood flow was recorded from 3 minutes before to 10 minutes after the endothelin infusion was begun. Thereafter, measurements were made at 5-minute intervals for 60 minutes. Blood pressure was measured 10 and 60 minutes after the infusion was begun.

TABLE 1. Mean Arterial Pressure, Heart Rate, and Forearm Blood Flows Before and After Brachial Artery Administration of Peptides in the Two Study Protocols (1 and 2) Comparing Endothelin-1 and Endothelin-3

	Protocol 1				Protocol 2	
Parameter	ET-1 (1 pmol)	ET-3 (1 pmol)	ET-1 (5 pmol)	ET-3 (5 pmol)	ET-1 (60 pmol)	ET-3 (60 pmol)
MAP, mm Hg						
Basal	89±5	88±6	90±5	88±4	87±5	83±4
10 min	...	...	...	...	89±6	87±3
60 min	91±6	89±7	89±4	86±4	89±6	90±4
HR, bpm						
Basal	65±4	66±3	67±5	66±4	66±2	66±2
10 min	...	...	...	...	63±4	65±3
60 min	71±6	67±4	68±6	62±5	64±5	65±2
Infused FBF, $mL \cdot 100\ mL^{-1} \cdot min^{-1}$						
Basal	3.9±0.9	4.0±0.7	3.9±0.9	4.4±1.3	3.7±0.6	3.0±0.3
60 min	3.4±0.9	3.7±0.8	3.1±1.0	3.3±0.5	3.2±0.4	2.4±0.3
Noninfused FBF, $mL \cdot 100\ mL^{-1} \cdot min^{-1}$						
Basal	3.0±0.5	3.2±0.5	3.0±0.5	4.1±0.6	3.4±0.7	3.3±0.2
60 min	2.8±0.5	3.1±0.6	3.9±0.9	4.0±1.0	3.6±0.6	3.7±0.6

ET indicates endothelin; MAP, mean arterial pressure; HR, heart rate; bpm, beats per minute; and FBF, forearm blood flow. Values are mean±SEM. There were no significant differences in basal MAP, HR, and FBF between study days. MAP, HR, and FBF in the noninfused arm did not change significantly on any study day after infusion of peptides.

Protocol 3: Intra-arterial endothelin-1 and sarafotoxin S6c. On two separate occasions, in random, balanced order, six subjects received endothelin-1 and sarafotoxin S6c via the brachial artery at 5 pmol/min for 60 minutes. Forearm blood flow was recorded from 3 minutes before to 5 minutes after peptide infusion was begun. Thereafter, measurements were made at 5-minute intervals for 60 minutes. Blood pressure was measured at 60 minutes, just before the infusion was terminated.

Hand Vein Studies

Protocol 4: Intravenous endothelin-1 and sarafotoxin S6c. Six subjects were studied on two separate occasions, in random, balanced order. Subjects rested semirecumbent throughout. The dorsal hand vein cannula and the LVDT were sited. Saline was infused for 30 minutes, during which vein diameter was measured every 5 minutes. Endothelin-1 and sarafotoxin S6c were infused at 5 pmol/min for 60 minutes, with measurements of vein diameter every 5 minutes. The choice of this dose was based on previous work that showed, in vivo, that endothelin-1 at 5 pmol/min causes slow-onset venoconstriction of ≈60% in human skin capacitance vessels.[29,31] Blood pressure was measured twice before the dose was given and at 60 minutes, just before the infusion was terminated.

Data Analysis and Statistics

Plethysmographic data listings were extracted from the CHART data files, and forearm blood flows were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (EXCEL 4.0; Microsoft Ltd). Because wrist cuff inflation results in a transient forearm vasoconstriction,[37] recordings made in the first 60 seconds after wrist cuff inflation were not used for analysis. Usually, the last five flow recordings in each 3-minute measurement period were calculated and averaged for the infused and noninfused arms. However, to detect early transient changes in blood flow, every recording made immediately before and after the start of peptide infusion was analyzed. Basal blood flow was taken as the average of all flow recordings made in the 2 minutes before infusion of peptides was begun. The intersubject, intrasubject (interstudy), and intrasubject (intrastudy) coefficients of variation for basal forearm blood flow measurements in our laboratory are 51%, 33%, and 14%, respectively. The intersubject, intrasubject (interstudy), and intrasubject (intrastudy) coefficients of variation for the basal ratio of blood flow between infused and noninfused arms in our laboratory are 22%, 15%, and 8%, respectively. Therefore, to reduce the variability of blood flow data, the ratio of flows in the two arms was calculated for each time point, in effect using the noninfused arm as a contemporaneous control for the infused arm.[38] Forearm blood flow results are shown as a percentage change from basal in the ratio of blood flow between infused and noninfused arms.[29]

Basal vein diameter was calculated as the mean of the last three measurements before the start of the peptide infusion, expressed in millimeters. The intersubject, intrasubject (interstudy), and intrasubject (intrastudy) coefficients of variation for basal hand vein diameter measurements in our laboratory are 43%, 26%, and 5%, respectively. Given the high intersubject and interstudy variability in hand vein diameter, responses after infusion of peptides are expressed as percentage change in vein diameter from basal.[32] Duplicate blood pressure measurements were averaged at each time point. Basal blood pressure was taken as the average of the second set of measurements made before infusion of peptides.

To obtain an estimate of the contribution of ET_B receptors to vasoconstriction, the ratio of constriction to the ET_B agonist compared with constriction to endothelin-1 was calculated for each subject at the 60-minute time point. Because these data had a skewed distribution, ratios were logarithmically transformed for statistical analysis. Data are shown as mean values, with 95% confidence intervals (CI) shown in the text and SEM in the figures. Data were examined by a repeated-measures ANOVA with statistical testing of overall significance by Scheffé's F test (ANOVA) using STATVIEW 512+ software (Brainpower Inc) for the Apple Macintosh personal computer.

Results

Basal blood pressure, heart rate, forearm blood flow, and vein diameter were similar on the different study days, and there was no significant difference in basal forearm blood flow between the infused and noninfused arms (Tables 1 and 2). Blood pressure, heart rate, and blood flow in the noninfused arm did not change significantly after infusion of any study agent, confirming that drug effects were confined to the infused arm (Tables 1 and 2).

TABLE 2. Mean Arterial Pressure, Heart Rate, Forearm Blood Flow, and Hand Vein Size Before and After Administration of Peptides in the Two Study Protocols (3 and 4) Comparing Endothelin-1 and Sarafotoxin S6c

Parameter	Protocol 3		Protocol 4	
	ET-1 (5 pmol IA)	S6c (5 pmol IA)	ET-1 (5 pmol IV)	S6c (5 pmol IV)
MAP, mm Hg				
Basal	82±3	82±3	89±5	84±3
60 min	85±3	85±4	89±4	84±2
HR, bpm				
Basal	62±8	63±3	70±4	65±4
60 min	55±3	64±4	68±5	59±5
Infused FBF, mL · 100 mL^{-1} · min^{-1}				
Basal	3.1±0.4	3.1±0.5	...	...
60 min	1.4±0.3	1.8±0.3	...	...
Noninfused FBF, mL · 100 mL^{-1} · min^{-1}				
Basal	2.7±0.4	2.7±0.4	...	...
60 min	2.3±0.2	2.3±0.5	...	...
Vein size, mm				
Basal	...	...	0.37±0.08	0.44±0.08

IA indicates intra-arterial; IV, intravenous. Other abbreviations as in Table 1. There were no significant differences in basal MAP, HR, FBF, and hand vein diameter between study days. MAP, HR, and FBF in the noninfused arm did not change significantly after infusion of peptides.

Protocol 1: Low-Dose Intra-arterial Endothelin-1 and Endothelin-3

Endothelin-1 at 1 pmol/min caused a modest but significant forearm vasoconstriction, with an 11% reduction in forearm blood flow at 60 minutes (CI, −22% to −1%; ANOVA, P=.02; Fig 1). Endothelin-3 at 1 pmol/min tended to decrease forearm blood flow, with a 5% reduction in blood flow at 60 minutes, but this was not significant (CI, −14% to +3%; ANOVA, P=.163; Fig 1). There was no significant difference between the responses to endothelin-1 and endothelin-3 at 1 pmol/min (ANOVA, P=.454). There was no significant vasodilatation early in the course of infusion of either peptide. The average ratio of forearm vasoconstriction to endothelin-3 and endothelin-1 was 0.16, although this estimate had wide CIs (CI, 0.03 to 0.98).

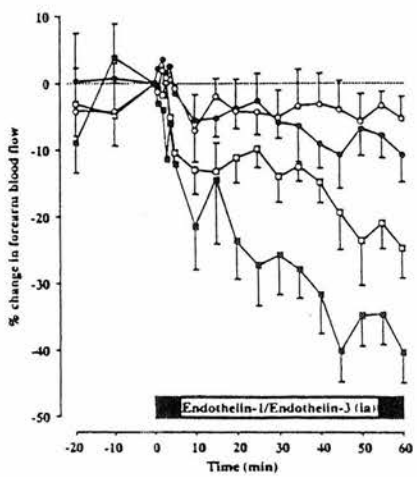

FIG 1. Graph. Six subjects received brachial artery infusion of endothelin-3 (○, 1 pmol/min; □, 5 pmol/min) and on a separate occasion, endothelin-1 (●, 1 pmol/min; ■, 5 pmol/min), each for 60 minutes. Shaded bar indicates period of infusion of endothelin isopeptides. Endothelin-1 caused significant forearm vasoconstriction at both doses, whereas the effect of endothelin-3 was significant only at 5 pmol/min. For clarity, error bars have been removed from that part of the figure showing results for the first 5 minutes of peptide infusion. ia indicates intra-arterial.

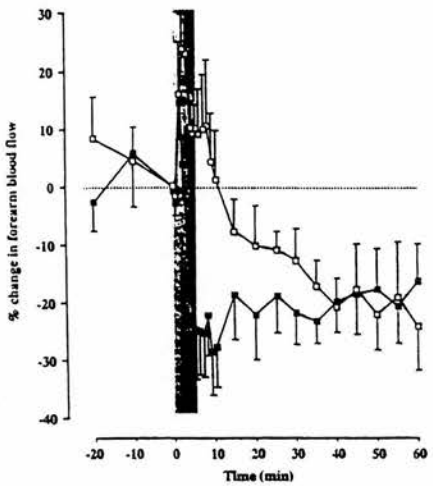

FIG 2. Graph. Six subjects received brachial artery infusion of endothelin-1 (■, 60 pmol/min) and on a separate occasion, endothelin-3 (□, 60 pmol/min), each for 5 minutes. Shaded area indicates period of infusion of endothelin isopeptides. Forearm vasodilatation occurred initially with endothelin-3 but not with endothelin-1. Both isopeptides then caused vasoconstriction of similar degree. ia indicates intra-arterial.

Endothelin-1 at 5 pmol/min caused substantial forearm vasoconstriction, with a 40% reduction in forearm blood flow at 60 minutes (CI, −52% to −28%; ANOVA, P=.0002; Fig 1). The same dose of endothelin-3 also significantly reduced forearm blood flow, with a 25% reduction in blood flow at 60 minutes (CI, −36% to −13%; ANOVA, P=.001; Fig 1). There was significantly greater vasoconstriction after endothelin-1 than endothelin-3 (ANOVA, P=.04). There was no significant vasodilatation early in the course of infusion of either peptide. The average ratio of forearm vasoconstriction to endothelin-3 and endothelin-1 was 0.58 (CI, 0.39 to 0.87).

Protocol 2: High-Dose Intra-arterial Endothelin-1 and Endothelin-3

Endothelin-1, at 60 pmol/min for 5 minutes, caused a trend to transient nonsignificant forearm vasodilatation in the first 2 minutes of infusion, with a maximum increase of 16% (CI, −7% to +23%; Fig 2) at 2 minutes. Thereafter, vasoconstriction occurred, with the maximum decrease in blood flow occurring at 10 minutes (−28%; CI, −48% to −9%), although flow was still reduced after 60 minutes (−17%; CI, −30% to −4%). Endothelin-3 caused significant early forearm vasodilatation, with a maximum increase in flow of 24% at 3 minutes (CI, +4% to +43%; Fig 2). Forearm vasoconstriction occurred after 10 minutes, with a maximum reduction in blood flow of 24% at 60 minutes (CI, −43% to −5%). There was a significant difference between the overall responses to endothelin-1 and endothelin-3 over the 60 minutes after bolus administration of isopeptide (ANOVA, P=.04). However, maximum vasoconstriction to the isopeptides was similar (Fig 2). The average ratio of forearm vasoconstriction to endothelin-3 and endothelin-1 was 0.82, although this estimate had wide CIs (CI, 0.13 to 5.07).

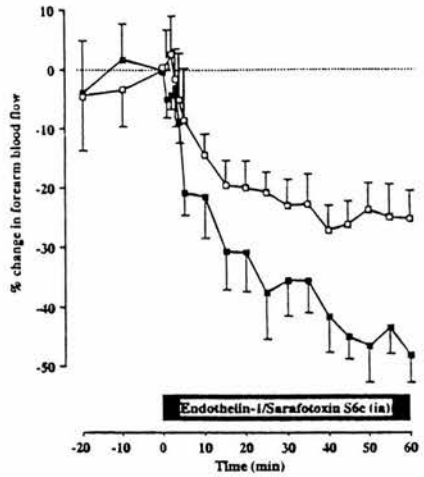

FIG 3. Graph. Six subjects received brachial artery infusion of endothelin-1 (■, 5 pmol/min) and on a separate occasion, sarafotoxin S6c (□, 5 pmol/min), each for 60 minutes. Shaded bar indicates period of infusion of peptides. Both peptides caused significant forearm vasoconstriction, although the effect of sarafotoxin S6c was less than that of endothelin-1. ia indicates intra-arterial.

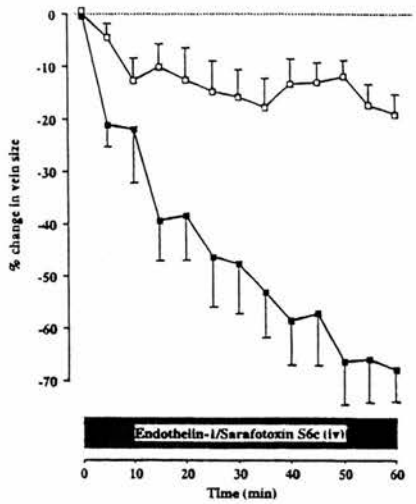

FIG 4. Graph. Six subjects received dorsal hand vein infusion of endothelin-1 (■, 5 pmol/min) and on a separate occasion, sarafotoxin S6c (□, 5 pmol/min), each for 60 minutes. Shaded bar indicates period of infusion of peptides. Both peptides caused significant venoconstriction, although the effect of sarafotoxin S6c was less than that of endothelin-1. iv indicates intravenous.

Protocol 3: Intra-arterial Endothelin-1 and Sarafotoxin S6c

Endothelin-1 at 5 pmol/min did not cause early vasodilatation but did produce slow-onset forearm vasoconstriction, with a maximum reduction in forearm blood flow of 48% at 60 minutes (CI, −60% to −37%; ANOVA, $P=.0001$; Fig 3). There was no significant vasodilatation to sarafotoxin S6c early in the course of the infusion, although there may have been a trend for this to occur (Fig 3). Like endothelin-1, sarafotoxin S6c caused slow-onset forearm vasoconstriction (ANOVA versus basal, $P=.002$; Fig 3). However, the maximum change in blood flow with sarafotoxin S6c at 60 minutes (−25%; CI, −37% to −13%) was significantly less than that to endothelin-1 (ANOVA, $P=.04$). The average ratio of forearm vasoconstriction to sarafotoxin S6c and endothelin-1 was 0.48 (CI, 0.30 to 0.75).

Protocol 4: Intravenous Endothelin-1 and Sarafotoxin S6c

Endothelin-1 caused a slow-onset and marked decrease in hand vein diameter, with a maximal reduction at 60 minutes (−68%; CI, −84% to −52%; ANOVA, $P=.001$; Fig 4). Sarafotoxin S6c also caused venoconstriction, although the maximum decrease in hand vein size at 60 minutes (−19%; CI, −29% to −9%; ANOVA versus basal, $P=.003$; Fig 4) was significantly less than that to endothelin-1 (ANOVA, $P=.002$). The average ratio of venoconstriction to sarafotoxin S6c and endothelin-1 was 0.25 (CI, 0.14 to 0.44).

Discussion

These studies show that selective agonists at endothelin ET_B receptors constrict forearm resistance and hand capacitance vessels in vivo in humans. In addition, high doses of endothelin-3, and perhaps of endothelin-1, cause transient forearm vasodilatation. These results suggest an important role for ET_B receptors in mediating the vascular effects of endothelin-1. It is possible that different findings might have been obtained if other vascular beds had been studied. However, responses in human forearm resistance vessels and dorsal hand veins are thought to be broadly representative of responses in other resistance and capacitance beds.[39] Given that resting forearm blood flow is ≈50 mL/min,[39] doses of 1, 5, and 60 pmol/min of peptide should achieve local concentrations of ≈0.02, ≈0.1, and ≈1 nmol/L, respectively. Endothelin-1 would be expected to act equally on both ET_A and ET_B receptors at these concentrations, while endothelin-3 would be expected to be relatively selective for the ET_B receptor, because this isopeptide has a K_i at ET_A receptors of about 140 nmol/L.[5] Sarafotoxin S6c at 5 pmol/min should have been highly selective for the ET_B receptor, because the calculated concentration in forearm blood (0.1 nmol/L) is at least 70 000-fold lower than its K_i at ET_A receptors (>7300 nmol/L).[5]

Administration of endothelin-3 at 60 pmol/min caused significant early forearm vasodilatation, and there was also a tendency for similar transient vasodilatation to occur with endothelin-1, although this was not statistically significant. Vasodilatation is likely to have been due to activation of ET_B receptors on endothelial cells, causing generation of endothelium-derived dilator substances.[31] The apparent absence of significant vasodilatation to high-dose endothelin-1 may have been due to additional early vasoconstriction mediated by ET_A receptors masking dilatation, although it should be noted that the CIs at these time points were sufficiently wide for an ≈20% vasodilatation to endothelin-1 to have been missed by chance. Lower doses of endothelin-1 and sarafotoxin S6c failed to cause early vasodilatation. The lack of vasodilatation to endothelin-1 contrasts with previous findings,[40] possibly because of differences in doses used and experimental design. In view of the relatively high doses required to cause vasodilatation, it is likely that vasodilatation to the endothelins represents a pharmacological rather than a physiological phenomenon. Because human dorsal hand veins have no basal tone, it is not possible to demonstrate whether endothelin-1 or sarafotoxin S6c causes venodilatation without

preconstriction of the vein. Previous work has shown no venodilatation to endothelin-1 or endothelin-3 in preconstricted dorsal hand veins.[41] Nonetheless, inhibition of prostaglandin but not nitric oxide generation potentiates venoconstriction to endothelin-1 in vivo in humans.[31] Thus, the venous endothelium may generate vasodilator substances in response to endothelin, but the vasodilator effects of such substances appear to be masked by the simultaneous direct venoconstriction caused by the peptide and serve only to modulate venoconstriction.

Given that both endothelin-3 and sarafotoxin S6c caused vasoconstriction, our results suggest the presence of vasoconstrictor ET_B receptors. However, constriction to the ET_B agonists was almost always less than that to the nonselective ET_A and ET_B agonist endothelin-1, implying that both ET_A and ET_B receptors contribute to vasoconstriction. The 95% CIs of the ratio of forearm vasoconstriction to sarafotoxin S6c and endothelin-1 are consistent with ET_B receptors contributing substantially to constriction, accounting for between 30% and 75% of the response to endothelin-1. Although the magnitude of the ET_B contribution in vitro appears to differ between vessels,[17] the similarity of responses in forearm resistance vessels and cutaneous capacitance vessels of the hand suggests that ET_B receptors may be of widespread functional importance in human blood vessels.

Our finding of ET_B receptor–mediated vasoconstriction of resistance vessels contrasts with some in vitro studies that suggest little contribution of ET_B receptors to constriction of human arteries.[11,17-24] This difference may reflect the fact that we examined responses in an intact resistance bed, because ET_B receptor–mediated vasoconstriction appears to play a relatively greater role in smaller vessels, particularly those responsible for determining resistance.[17,42] All of the in vitro studies in which human vessels exhibited little or no ET_B–mediated arterial vasoconstriction examined vessels >400 μm in diameter.[11,17-24] In addition to the influence of vessel size on the contribution of ET_B receptors, there may be regional differences. Local injection of the ET_A antagonist PD147953 has been shown to completely prevent vasoconstriction of human skin vessels caused by intradermal injection of endothelin-1, suggesting that vasoconstriction is mediated mainly by ET_A receptors in this microvascular bed.[43] The effects of sarafotoxin S6c, compared with those of endothelin-1, were less in hand veins than in forearm resistance vessels, despite in vitro evidence from animal vessels that responses to ET_B agonists are greater in veins than arteries.[8,9,12,13] This may reflect a true species difference, because endothelin-1 is about eightfold more potent as a venoconstrictor than endothelin-3 in human hand veins,[41] which also suggests that ET_A receptors predominate in these vessels.

Although vasoconstriction to the ET_B agonists endothelin-3 and sarafotoxin S6c is most likely to be caused by stimulation of vascular smooth muscle ET_B receptors, there are alternative explanations. First, ET_B receptors may be confined to endothelial cells but cause late-onset vasoconstriction through stimulation of the generation of endothelium-derived vasoconstrictor agents. These substances might include constrictor prostanoids or even endothelin-1, because endothelin-3 is known to stimulate production of endothelin-1 in vitro.[44] Second, some of the effects of endothelin-3 could have been mediated by a putative ET_C (endothelin-3–selective) receptor situated on endothelial cells. However, although there is evidence from binding[44] and functional[45] studies to support the existence of an endothelin-3–selective receptor in the vasculature, and a potential candidate has been identified in *Xenopus laevis* melanophores,[46] this receptor has not been identified in humans. Any contribution from the putative ET_C receptor will depend on its isolation and pharmacological characterization. Third, there may be receptor-mediated clearance of endogenously generated endothelin-1 by ET_B receptors, as has been shown in animals.[47] If this were the case, ET_B agonists might prevent local clearance of endothelin-1, which would then act on ET_A receptors to cause vasoconstriction. However, this possibility appears highly unlikely, given that ET_A antagonists do not influence vasoconstriction to sarafotoxin S6c in vitro.[8,10,13,26,48] In future, studies with selective ET_B receptor antagonists should clarify this issue, because such agents would be expected to potentiate responses to endothelin-1 if ET_B receptor–mediated clearance of endothelin-1 does occur.

Thus, the most likely explanation for our results is that there are functionally active ET_A and ET_B receptors on vascular smooth muscle cells causing vasoconstriction, to both of which endothelin-1 would have access. These findings have implications for the future development of antiendothelin therapies, because they suggest that full inhibition of vasoconstriction to endogenously generated endothelin-1 may be obtained only by use of either combined $ET_{A/B}$ endothelin receptor antagonists[49] or inhibitors of endothelin generation.[29]

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Forearm Vasoconstriction to Endothelin-1 Is Mediated by ET_A and ET_B Receptors In Vivo in Humans

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Summary: The role of endothelin (ET)-B (ET_B) receptors in mediating vasoconstriction in humans is unclear. As yet, in vitro data have been contradictory, and there have been no in vivo studies in resistance vessels. We investigated the function of ET_B receptors in vivo in human forearm resistance vessels using ET-1 as a nonselective agonist at ET_A and ET_B receptors and ET-3 and sarafotoxin S6c as ET_B receptor agonists. Brachial artery infusion of ET-1 and ET-3 caused slow-onset, dose-dependent forearm vasoconstriction. Although ET-3 caused significantly less forearm vasoconstriction than ET-1 at low doses, vasoconstriction to the two isopeptides was similar at the highest dose (60 pmol/min). ET-3 caused initial transient forearm vasodilatation at this dose, whereas ET-1 showed only a nonsignificant trend toward causing early vasodilatation. Intra-arterial sarafotoxin S6c caused a progressive reduction in forearm blood flow, although less than that to ET-1. Therefore, ET_B receptor agonists contract human resistance vessels in vivo. The effects of ET-3 and sarafotoxin S6c, compared with ET-1, suggest that both ET_A and ET_B receptors mediate vasoconstriction. Antagonists at both ET_A and ET_B receptors, or inhibitors of the generation of ET-1, may be necessary to completely prevent vasoconstriction to endogenously generated ET-1. **Key Words:** Endothelins—Sarafotoxin S6c—Vasoconstriction—Vasodilatation.

Endothelin (ET)-1 was initially believed to mediate vasoconstriction solely through vascular smooth-muscle cell ET_A receptors, and ET_B receptors were believed to mediate only endothelium-dependent dilation (1,2). More recent evidence suggests that ET_B receptor mRNA is expressed in human vascular smooth muscle obtained from the aorta, pulmonary artery, and coronary artery (3), consistent with a vasoconstrictor role for this receptor. Indeed, in animals there is functional evidence for ET_B receptor–mediated vasoconstriction in vitro and in vivo (4–6), although the contribution is variable and appears to depend markedly on species, vessel type, and vessel size (3). Furthermore, the functional significance of such vascular smooth muscle ET_B receptors in humans is unclear, with apparently contradictory results being obtained from in vitro studies (7,8).

In view of the inconsistent results with in vitro studies, we have investigated the function of ET_A and ET_B receptors in blood vessels in vivo in human subjects. We used ET-1 as a nonselective agonist at ET_A and ET_B receptors, and ET-3 and sarafotoxin S6c as selective ET_B receptor agonists, these peptides having respectively about 2,000- and >30,000-fold selectivity for the ET_B over the ET_A receptor (9). These agents were administered via the brachial artery and forearm resistance vessel responses were assessed by plethysmography. We used local doses of peptides so that interpretation of the results would not be confounded by direct effects of systemic administration on kidney, heart, or brain, or by reflex effects consequent on changes in blood pressure.

MATERIALS AND METHODS

Subjects

Eighteen healthy male subjects between 22 and 38 years of age participated in these studies, which were conducted with the approval of the local Ethics Committee and with the written informed consent of each subject. All studies were performed in a quiet room maintained at a constant temperature of 22–25°C.

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Procedures

All drugs were administered via a brachial artery as previously described (10). Blood flow was measured in both forearms by venous occlusion plethysmography using indium/gallium-in-silastic strain gauges and blood pressure measured in duplicate in the noninfused arm as before (10).

Study design

Subjects rested recumbent throughout each study. Strain gauges and arm cuffs were applied and the left brachial artery cannula sited. Saline was infused for 30 min, during which two measurements of forearm blood flow were made (at −20 and −10 min). Three protocols were then followed, each in separate groups of subjects.

Protocol 1: low-dose intra-arterial ET-1 and ET-3. On four separate occasions, in random order, six subjects received ET-1 (Clinalfa, NovaBiochem, Nottingham, U.K.) and ET-3 (Clinalfa) at 1 and 5 pmol/min, each for 60 min. Forearm blood flow was recorded from 3 min before to 5 min after starting ET. Thereafter, measurements were made at 5-min intervals for 60 min. Blood pressure was measured 60 min after starting the infusion.

Protocol 2: high-dose intra-arterial ET-1 and ET-3. On two separate occasions, in random order, six subjects received ET-1 and ET-3 at 60 pmol/min for 5 min, followed by saline for 55 min. Forearm blood flow was recorded from 3 min before to 10 min after starting ET. Thereafter, measurements were made at 5-min intervals for 60 min. Blood pressure was measured 10 and 60 min after starting the infusion.

Protocol 3: intra-arterial ET-1 and sarafotoxin S6c. On two separate occasions, in random, balanced order, six subjects received ET-1 and sarafotoxin S6c (Sigma Chemical Co., Ltd., Poole, Dorset, U.K.) at 5 pmol/min for 60 min. Forearm blood flow was recorded from 3 min before to 5 min after starting peptide infusion. Thereafter, measurements were made at 5-min intervals for 60 min. Blood pressure was measured at 60 min, just before halting the infusion.

Data analysis and statistics

Basal blood flow was taken as the average of all flow recordings made in the 2 min before starting infusion of peptides. Forearm blood flow results are shown as a percentage change from basal in the ratio of blood flow between infused and noninfused arms (10). Duplicate blood pressure measurements were averaged at each timepoint. To obtain an estimate of the contribution of ET_B receptors to vasoconstriction, the ratio of constriction to the ET_B agonist compared with constriction to ET-1 was calculated for each subject at the 60-min time point. Because these data had a skewed distribution, ratios were log transformed for statistical analysis and are expressed as means with 95% confidence intervals (CI). Other data are shown as mean values with SEM. Data were examined statistically by a repeated measures analysis of variance combined with Scheffe's F test.

RESULTS

Protocol 1: low-dose intra-arterial ET-1 and ET-3

ET-1 at 1 pmol/min caused modest but significant forearm vasoconstriction, with a 11 ± 4% reduction in forearm blood flow at 60 min ($p = 0.02$). ET-3 at 1 pmol/min tended to decrease forearm blood flow, with a 5 ± 3% reduction in blood flow at 60 min, but this was not significant ($p = 0.163$). The average ratio of forearm vasoconstriction to ET-3 and ET-1 at 1 pmol/min was 0.16, although this estimate had wide confidence intervals (CI 0.03–0.98). ET-1 at 5 pmol/min caused substantial forearm vasoconstriction, with a 40 ± 5% reduction in forearm blood flow at 60 min ($p = 0.0002$). The same dose of ET-3 also significantly reduced forearm blood flow, with a 25 ± 5% reduction in blood flow at 60 min ($p = 0.001$). The average ratio of forearm vasoconstriction to ET-3 and ET-1 at 5 pmol/min was 0.58 (CI 0.39–0.87). There was no significant vasodilation early in the course of infusion of either peptide, at either dose.

Protocol 2: high-dose intra-arterial ET-1 and ET-3

ET-1 at 60 pmol/min for 5 min caused transient nonsignificant forearm vasodilatation in the first 2 min of infusion, followed by vasoconstriction, with the maximal decrease in blood flow occurring at 10 min, although flow was still reduced after 60 min (Fig. 1). ET-3 caused significant early forearm vasodilatation, followed by slow-onset vasoconstriction. There was a significant difference between the overall responses to ET-1 and ET-3 over the 60 min after bolus administration of isopeptide ($p = 0.04$), although maximum vasoconstriction to the isopeptides was similar (Fig. 1). The average ratio of fore-

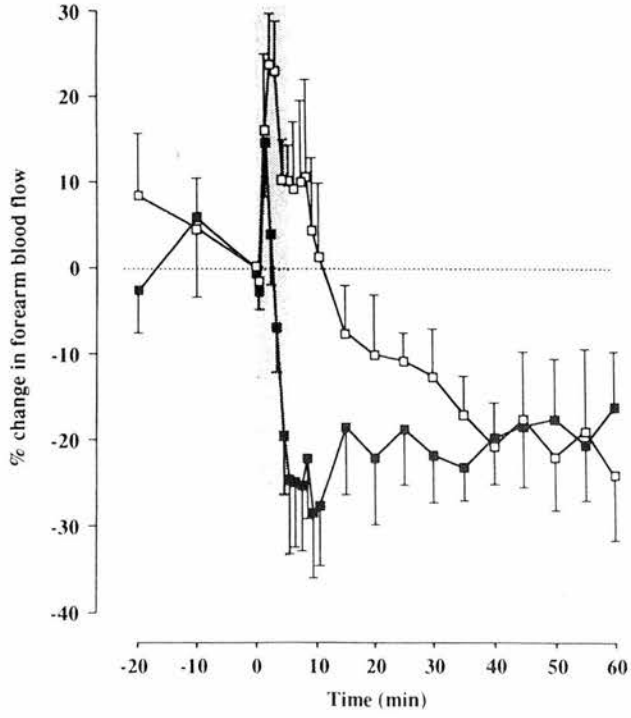

FIG. 1. Six subjects received brachial artery infusion of ET-1 (■, 60 pmol/min), and, on a separate occasion, ET-3 (□, 60 pmol/min), each for 5 min. The shaded area indicates the period of infusion of endothelin isopeptides. Forearm vasodilatation occurred initially with ET-3 but not with ET-1. Both isopeptides then caused vasoconstriction of similar degree.

arm vasoconstriction to ET-3 and ET-1 was 0.82 (CI 0.13–5.07).

Protocol 3: intra-arterial ET-1 and sarafotoxin S6c

ET-1 produced slow-onset forearm vasoconstriction ($p = 0.0001$) (Fig. 2). There was no significant vasodilation to sarafotoxin S6c early in the course of the infusion, although there may have been a trend for this to occur (Fig. 2). Like ET-1, sarafotoxin S6c caused slow-onset forearm vasoconstriction ($p = 0.002$). However, the maximum change in blood flow with sarafotoxin S6c at 60 min was significantly less than that to ET-1 ($p = 0.04$). The average ratio of forearm vasoconstriction to sarafotoxin S6c and ET-1 was 0.48 (CI 0.30–0.75).

Basal blood pressure, heart rate, and forearm blood flow were similar on the different study days, and there was no significant difference in basal forearm blood flow between the infused and noninfused arms. Blood pressure, heart rate, and blood flow in the noninfused arm did not change significantly after infusion of any study agent, confirming that drug effects were confined to the infused arm.

DISCUSSION

These studies show that selective agonists at ET_B receptors constrict forearm resistance vessels in vivo in humans. In addition, high doses of ET-3, and perhaps of ET-1, cause transient forearm vasodilatation. These findings suggest a potentially important role for ET_B receptors in mediating the vascular effects of ET-1. Given that resting forearm blood flow is ~50 ml/min, doses of 1, 5, and 60 pmol/min of peptide should achieve local concentrations of ~0.02, ~0.1, and ~1 n*M*, respectively. ET-1 would be expected to act equally on both ET_A and ET_B receptors at these concentrations, whereas ET-3 would be expected to be relatively selective for the ET_B receptor, because this isopeptide has a K_i at ET_A receptors of approximately 140 n*M* (9). Sarafotoxin S6c at 5 pmol/min should have been highly selective for the ET_B receptor because the calculated concentration in forearm blood (0.1 n*M*) is at least 70,000-fold lower than its K_i at ET_A receptors (>7,300 n*M*) (9).

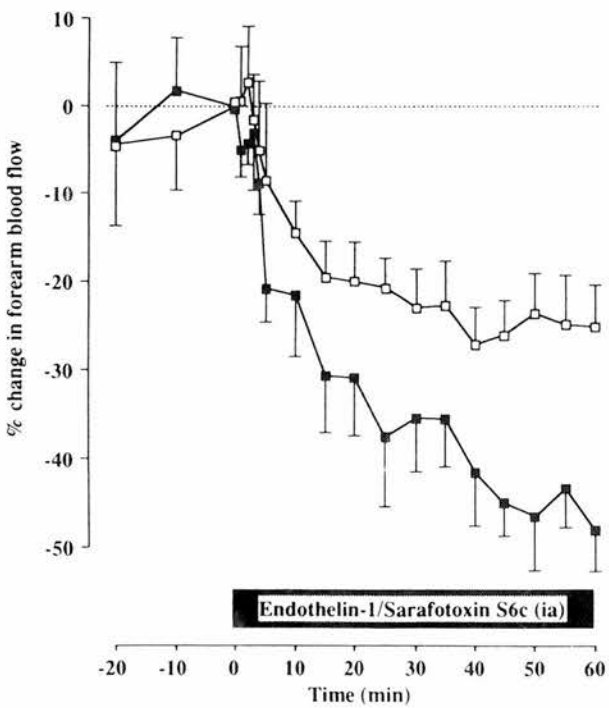

FIG. 2. Six subjects received brachial artery infusion of endothelin-1 (■, 5 pmol/min), and, on a separate occasion, sarafotoxin S6c (□, 5 pmol/min), each for 60 min. The shaded bar indicates the period of infusion of peptides. Both peptides caused significant forearm vasoconstriction, although the effect of sarafotoxin S6c was less than that of ET-1 (ia, intra-arterial).

Administration of ET-3 at 60 pmol/min caused significant early forearm vasodilation, and there was also a tendency for similar transient vasodilation to occur with ET-1, though it was not statistically significant. Vasodilation is likely to have been due to activation of ET_B receptors on endothelial cells, causing generation of ET-derived dilator substances (2). The apparent absence of significant vasodilation to high-dose ET-1 may have been due to additional early vasoconstriction mediated by ET_A receptors, thus masking dilation. Lower doses of ET-1 and sarafotoxin S6c failed to cause early vasodilation. In view of the relatively high doses required to cause vasodilation, it is likely that vasodilation to the endothelins represents a pharmacologic rather than a physiologic phenomenon.

Given that both ET-3 and sarafotoxin S6c caused vasoconstriction, our results indicate the presence of vasoconstrictor ET_B receptors. However, this constriction was less than that to ET-1, implying that both ET_A and ET_B receptors contribute to the vasoconstriction. The 95% confidence intervals of the ratio of forearm vasoconstriction to sarafotoxin S6c and ET-1 are consistent with ET_B receptors contributing 30–75% of the response to ET-1. Our finding of vasoconstrictor ET_B receptors in human resistance vessels contrasts with some in vitro studies (3). This difference may reflect the fact that we examined responses in an intact resistance bed, because ET_B receptor–mediated vasoconstriction appears to play a relatively greater role in the smaller vessels that determine resistance (3,11).

Although the vasoconstriction to ET_B agonists is most likely caused by stimulation of vascular smooth muscle ET_B receptors, there are alternative explanations. First, ET_B receptors may be confined to endothelial cells but cause late-onset vasoconstriction through stimulation of the generation of endothelium-derived vasoconstrictor agents. These substances might include constrictor prostanoids or even ET-1, because ET-3 is known to stimulate production of ET-1 in vitro (12). Second, some of the

effects of ET-3 could have been mediated by a putative ET_C (ET-3–selective) receptor, although as yet this has been identified only in *Xenopus laevis* melanophores (13). Third, there may be receptor–mediated clearance of endogenously generated ET-1 by ET_B receptors, as has been shown in animals (14). If this were the case, ET_B agonists might prevent local clearance of ET-1, which would then act on ET_A receptors to cause vasoconstriction. However, this possibility appears highly unlikely because ET_A antagonists do not influence vasoconstriction to S6c in vitro (8). In the future, studies with selective ET_B receptor antagonists should clarify this issue, because if ET_B receptor–mediated clearance of ET-1 does occur, such agents would be expected to potentiate response to ET-1.

In conclusion, the most likely explanation for our results is that there are functionally active ET_A and ET_B receptors on vascular smooth muscle cells causing vasoconstriction, to both of which ET-1 would have access. These findings have implications for the future development of anti-endothelin therapies, because they suggest that full inhibition of vasoconstriction to endogenously generated ET-1 can be obtained only with either combined $ET_{A/B}$ receptor antagonists or inhibitors of endothelin generation.

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Endothelium-Dependent Modulation of Venoconstriction to Sarafotoxin S6c in Human Veins In Vivo

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Summary: We investigated the vascular effects mediated by ET_A and ET_B receptors in human dorsal hand veins in vivo, using sarafotoxin S6c (SFTX6c) as a selective agonist of ET_B receptors and endothelin-1 (ET-1) as a nonselective agonist of ET_A and ET_B receptors. The cyclooxygenase inhibitor aspirin and the nitric oxide synthase inhibitor L-NMMA were used to examine the modulating role of endothelial vasodilators on the response to SFTX6c. Drugs were all infused into the hand veins, at locally but not systemically active doses, via a 23 SWG butterfly cannula, with the exception of aspirin, which was administered orally. Hand vein size was measured by the Aellig technique. The study was performed in six healthy male subjects. Data (mean ± SEM) were examined by ANOVA. Results are expressed as percent change from baseline at 60 min. ET-1 (5 pmol/min for 60 min) caused venoconstriction of 68 ± 6% ($p = 0.0001$). SFTX6c at the same dose caused venoconstriction of 19 ± 4% ($p = 0.003$). The response to SFTX6c was significantly less than to ET-1 ($p = 0.002$). Constriction to SFTX6c tended to increase when this agent was coadministered with aspirin (25 ± 7%) or L-NMMA (24 ± 10%) and was significantly potentiated when these agents were co-administered (45 ± 4%; $p = 0.01$ vs. SFTX6c alone). We have demonstrated that the selective ET_B agonist SFTX6c produces venoconstriction in human hand veins in vivo and that this venoconstriction is modulated by the generation of endothelium-derived vasodilators. In this vascular bed, venoconstriction rather than venodilatation appears to be the predominant effect of stimulation of ET_B receptors with SFTX6c. Key Words: Human—Venoconstriction—Endothelin-1—Sarafotoxin S6c—Endothelium.

The endothelins are a family of 21-amino-acid peptides with potent and characteristically sustained vasoconstrictor and vasopressor actions (1). Endothelin-1 (ET-1) is the predominant isopeptide generated by the vascular endothelium (2) and therefore appears to be the most important isoform mediating cardiovascular effects. Two specific receptors for the endothelins have been isolated by in vitro expression of cloned human cDNA (3,4). The ET_A receptor has a high affinity for ET-1 compared with endothelin-3 (ET-3), whereas the ET_B receptor has equal affinity for both endothelins.

Vasoconstriction to ET-1 was initially believed to be mediated solely by vascular smooth-muscle cell ET_A receptors, and endothelial cell ET_B receptors were believed only to mediate generation of endothelium-derived dilator substances. Indeed, there is considerable evidence that stimulation of endothelial ET_B receptors can cause production of nitric oxide and dilator prostanoids, both to cause the initial depressor effect of systemic bolus administration of ET-1 and to modulate the sustained vasoconstriction associated with ET-1 (5). More recently, it has been shown that ET_B receptor mRNA is expressed in human vascular smooth muscle (6), consistent with a potential vasoconstrictor role for the ET_B receptor. Indeed, in animals and in humans, there is functional evidence for ET_B receptor-mediated vasoconstriction in vitro, particularly in veins (7–9). However, the contribution of ET_B receptors to ET-1–mediated constriction can vary depending on species, vessel type, and vessel size.

In human hand veins, venoconstriction to ET-1 is attenuated by dilator prostanoids but not by nitric oxide (10). Here, we have examined the role of the endothelium-derived dilators nitric oxide and prostacyclin in modulating venoconstriction to locally but not systemically active doses of sarafotoxin S6c

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(SFTX6c), an endothelin receptor agonist with 30,000-fold selectivity for the ET_B receptor in vitro (11).

MATERIALS AND METHODS

Experimental procedures

Six healthy men (age range 25–39 years) participated in the study, with local Ethics Review Committee approval. Subjects rested semirecumbent in a quiet room maintained at a constant temperature for each study (25–27°C). Drugs were infused via a 23 SWG cannula (Abbott) sited in a selected dorsal hand vein in the direction of flow, as described previously (10). Local anesthesia was not employed. The same dorsal hand vein was used in each study. Internal diameter of the dorsal hand vein was measured by the Aellig displacement technique (12) at 5-min intervals throughout each study period. Blood pressure was measured in the noninfused arm at 30-min intervals.

Drugs

On separate occasions, ET-1 (NovaBiochem, Nottingham, U.K.) and SFTXS6c (Sigma Chemical Co Ltd., Nottingham, U.K.) were administered for 60 min at a constant rate of 5 pmol/min, based on results from previous studies (10,13). A single dose was used because the slow onset and long-lasting action of the endothelins precludes the use of repeated doses in a single study to examine conventional dose-response relationships. $L\text{-}N^G$-monomethyl-arginine (L-NMMA; NovaBiochem, Nottingham, U.K.), a specific substrate analogue inhibitor of nitric oxide synthase in humans (14), was administered in a dose of 100 nmol/min (10). This dose of L-NMMA has no effect on basal hand vein size (15). Aspirin (600 mg, soluble; Reckitt & Coleman, Hull, U.K.) was dissolved in 200 ml water and administered 30 min before local peptide infusions. Aspirin irreversibly inhibits cyclo-oxygenase (EC 1.14.99.1) and, when given at this dose, inhibits bradykinin-stimulated endothelial production of prostacyclin by at least 85%, with recovery developing over the next 6 h (16). All drugs, with the exception of aspirin, were dissolved in saline (0.9%; Travenol). In each study, saline was infused for 30 min before infusion of the study agent. The *total* rate of infusion was maintained constant throughout all studies at 0.25 ml/min.

Study design

The study involved five separate study periods in the same subjects, each study period separated by at least 1 week. Responses to ET-1 and SFTX6c alone were examined. In addition, responses to SFTX6c were investigated in the presence of aspirin and L-NMMA, administered both independently and together. The order of the study periods was randomized, with the exception of the sarafotoxin and combined L-NMMA and aspirin study, which was the final study period for each subject.

Data presentation and statistics

Basal vein size was calculated in millimeters as the mean of the last two measurements taken during saline infusion. Vein size during drug administration was expressed as percentage change in vein size from basal, at 60 min. All results are expressed as mean ± SEM. Data were examined by repeated measures analysis of variance. Statistical significance was accepted at the 5% level.

RESULTS

In the six subjects, basal vein size was similar for each study and blood pressure did not alter significantly throughout any of the study periods. ET-1 alone caused a slowly developing venoconstriction, with a maximum 68 ± 6% reduction of basal internal vein diameter at 60 min ($p = 0.0001$ vs. basal). SFTX6c also caused significant venoconstriction (19 ± 4%; $p = 0.003$ vs. basal). The response to ET-1 was substantially greater than that to SFTX6c ($p = 0.002$) (Fig. 1). Venoconstriction to SFTX6c tended to increase after administration of aspirin (25 ± 7%) and during co-infusion of L-NMMA (24 ± 10%), but was not significantly different than that to SFTX6c alone (Fig. 2). Venoconstriction to SFTX6c was substantially and significantly increased in the presence of aspirin and L-NMMA together (46 ± 9%; $p = 0.01$ vs. SFTX6c; $p = 0.0001$ vs. basal). Venoconstriction to SFTX6c in the presence of both aspirin and L-NMMA was less prominent than, but did not differ significantly from, that to ET-1 when infused alone ($p = 0.1$) (Figs. 1 and 2).

DISCUSSION

In these studies we have investigated the venoconstrictor effects of SFTX6c and their modulation by the endothelium-derived dilators nitric oxide and prostacyclin. We have confirmed the earlier findings that ET-1 (10) and SFTX6c (13) constrict human hand veins. As before (13), an equivalent dose of SFTX6c causes less venoconstriction than ET-1.

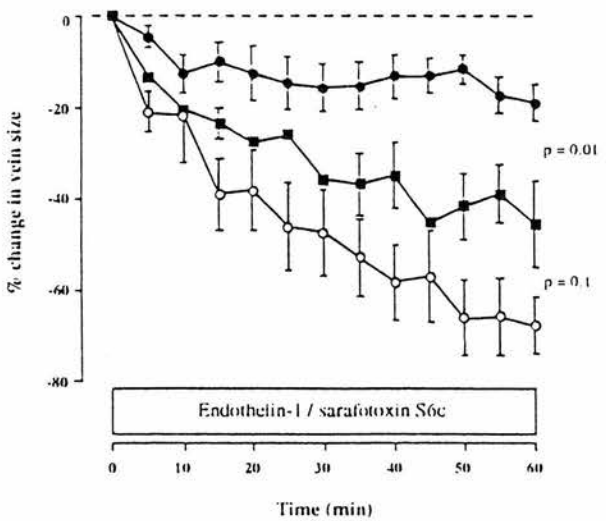

FIG. 1. Endothelin-1 (5 pmol/min; open circles), sarafotoxin S6c (5 pmol/min alone; closed circles), and sarafotoxin S6c (5 pmol/min with L-NMMA 100 nmol/min and after aspirin 600 mg; closed squares).

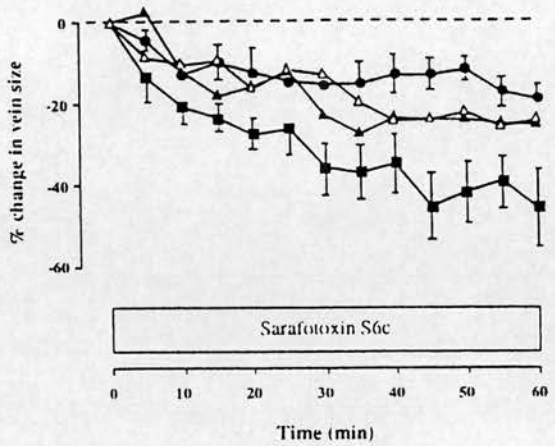

FIG. 2. Sarafotoxin S6c (5 pmol/min alone; closed circles), co-infused with L-NMMA (open triangles), after aspirin 600 mg (closed triangles), and with both L-NMMA and aspirin (closed squares).

These findings suggest that ET_B receptors contribute to but do not wholly account for ET-1-mediated venoconstriction.

In addition, we have demonstrated that the venoconstriction to SFTX6c is significantly and substantially increased when generation of both nitric oxide and dilator prostanoids, most likely prostacyclin, is blocked. Combined inhibition of nitric oxide and prostacyclin appeared to produce a greater effect on venoconstriction to SFTX6c than the addition of the individual effects of inhibition of generation of either nitric oxide or prostacyclin alone. This may reflect a capacity for the endothelium to compensate for inhibition of one dilator mediator by increased production of another. We have shown previously that responses to ET-1 are potentiated by aspirin administration but not by co-infusion of L-NMMA (10). It would be important to further investigate responses to ET-1 in human hand veins in vivo in the presence of L-NMMA and aspirin, both alone and given together. It appears from our results with SFTX6c that the endothelial ET_B receptor modulates the constrictor effects produced by stimulation of the vascular smooth muscle ET_B receptors, through generation of vasodilator substances by the endothelium. However, in these experiments the vasoconstrictor action predominates. In diseases such as chronic heart failure, in which endothelium-dependent vasodilatation is impaired, venoconstrictor effects of ET-1 may be enhanced as a result of unopposed vasoconstrictor effects mediated by both ET_A and ET_B receptors on vascular smooth muscle. Given that the overall effect of combined stimulation of the vascular smooth muscle and endothelial cell ET_B receptors is venoconstriction, it may be necessary to block both ET_A and ET_B receptors to completely block venoconstriction to ET-1. Drugs that would act specifically on the vascular smooth-muscle receptors (17) may be more effective vasodilators, because they would fully block ET-1 mediated vasoconstriction while allowing the potentially desirable effects of the endothelial ET_B receptors to be preserved.

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